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Contribuições para o conhecimento dos efeitos do BHT e outros antioxidantes no fígado

LORENA DOS SANTOS CASTRO

Maringá

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Dissertação apresentada ao programa de Pós Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de mestre em Ciência de Alimentos

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LORENA DOS SANTOS CASTRO

"CONTRIBUIÇÕES PARA O CONHECIMENTO DOS EFEITOS DO BHT E OUTROS ANTIOXIDANTES NO FÍGADO".

Dissertação apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-graduação em Ciência de Alimentos, para obtenção do grau de Mestre em Ciência de Alimentos.

Profa. Dra. Ana Carla Broetto Biazon

Profa. Dra. Cristiane Vizioli de Castro Ghizoni

Prof. Dr. Adelar Bracht
(Orientador)

Maringá - 2017

Orientador

Adelar Bracht

Co-Orientadora

Lívia Bracht

BIOGRAFIA

Lorena dos Santos Castro, brasileira, nascida em 19 de novembro de 1993, na cidade de Colorado, Paraná. Concluiu seu ensino médio no Colégio Estadual Monteiro Lobato de Colorado no ano de 2010. Em 2011 iniciou o curso de graduação em nutrição pela Uningá de Maringá, com conclusão em dezembro de 2014. No ano de 2015 iniciou o mestrado como aluna regular no programa de Pós-graduação em Ciência dos Alimentos- UEM. Durante essa jornada no Laboratório de Metabolismo Hepático, adquiriu experiência na área de bioquímica, atuando principalmente nos seguintes temas: metabolismo hepático, perfusão de fígado de rato e dosagens enzimáticas.

Dedico

Primeiramente a Deus, por guiar meus passos e ouvir minhas orações.

Aos meus pais amados, Carla e José Luiz, pelo apoio e incentivo durante toda a jornada de estudos.

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APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de dois artigos científicos:

1 Lorena dos Santos Castro, Lívia Bracht, Jurandir Fernando Comar, Rosane Marina Peralta and Adelar Bracht**, A reappraisal of the proposed metabolic and antioxidant actions of butylated hydroxytoluene (BHT) in the liver**. *Journal of Biochemical and Molecular Toxicology* (aceito).

2 Lorena dos Santos Castro, Lívia Bracht, Rosane Marina Peralta and Adelar Bracht. **Free radical scavenging activity of antioxidants in chemical systems and in isolated mitochondria** (ser submetido oportunamente).

GENERAL ABSTRACT

INTRODUCTION

Butylated hydroxytoluene (BHT) is amply utilized as an antioxidant in industrially processes foods, cosmetics, pharmaceutical and petrochemical products. It is also commercialized in the form of capsules as a food and health supplement. As antioxidant, BHT inhibits lipid peroxidation, an effect that it also exerts in biological systems. There is also a universal consensus that ingestion of molecular species able to scavenge free radicals (antioxidants) can, in part at least, prevent the deleterious effects of the reactive oxygen species (ROS). The mitochondrial ROS production can contribute significantly to damages to the organelles in several pathologies. For this reason, good free radical scavengers should be active at the lowest possible concentration, allowing the ingestion of lowest possible doses. The evaluation of the antioxidant activity of a given substance is usually assessed by chemical methods, such as those ones based on the capacity of scavenging free radicals generated by the compounds 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 1,1-diphenyl-2-picryl-hydrazyl (DPPH). There is obviously no guarantee that a given substance active in scavenging free radicals in an artificial system will also be able to scavenge with the same efficiency the reactive oxygen species produced in the mitochondria, for example. In addition to its effects as antioxidant, potential adverse effects of BHT were already reported. For example, there are studies showing that BHT labilizes the lisosomal membranes and disorganizes the mitochondrial structure, facts that could suggest that BHT interferes with cellular metabolism. In fact, there are reports that BHT affects mitochondrial functioning by, at least, two different mechanisms: uncoupling between electron transfer and oxidative phosphorylation and inhibition of complex I of the electron transport chain. This data suggest, in theory, that BHT should either stimulate or inhibit oxygen uptake, stimulate catabolic pathways and inhibit anabolic pathways. Such actions, beside others, have been clearly demonstrated to occur with several uncouplers and respiratory inhibitors.

AIMS

Taking into account what was exposed above, this work has two main objectives: 1) to conduct a systematic study on the effects of BHT on the rat liver, using both the isolated perfused rat liver and isolated mitochondria; 2) to compare the free radical scavenging activity of BHT and other well known antioxidants in chemical systems and in isolated mitochondria. Regarding the latter aspect, this study should clarify about the real antioxidant potential of BHT and several other antioxidants in a biological system (ROS generation in mitochondria).

MATERIALS AND METHODS

Male Wistar rats weighing 200–280 g were used in all experiments. All experiments were done in accordance with the worldwide accepted ethical guidelines for animal experimentation and were previously approved by the Ethics Committee of Animal Experimentation of the University of Maringá (protocol n. 9120290915). Isolated perfused rat liver experiments were done using hemoglobin-free, non-recirculating perfusion (Krebs/Henseleit-bicarbonate buffer, pH 7.4, saturated with O_2:CO_2 in the proportion of 95:5). BHT was dissolved into the perfusion fluid and infused in the concentration range of 1-500 μM. Lactate and fructose were used as gluconeogenic substrates. Samples of the perfusion fluid were collected and

enzymatically analysed for glucose, lactate and pyruvate. Oxygen uptake was continuously monitored by a platinum electrode. The mitochondrial respiratory activity was evaluated by two different assays. The first one was the classical assay in which intact phosphorylating mitochondria were used. Succinate was used as the substrate and the rate of oxygen uptake in the presence (state III) or in the absence (state IV) of ADP was measured, as well as the respiratory control ratio (RC). The final BHT concentrations in this assay were in the range between 10 and 100 μM. Besides this, assays with non-phosphorylating mitochondria were also performed, with the addition of oligomycin (a classical inhibitor of ATP synthase). Succinate was also used as the substrate and BHT was tested in the range between $2x10^{-9}$ to 10^{-4} M. The effects of BHT and 13 other antioxidants on ROS production in mitochondria were also investigated. The rate of mitochondrial ROS production was estimated by measuring the linear fluorescence increase (504 nm for excitation and 529 nm for emission) due to 2'-7'-dichlorofluorescein (DCF) formation from the reduced form of 2'-7'-dichlorofluorescein (DCFH) via oxidation by H_2O_2 in the presence of horseradish peroxidase, using succinate as substrate. Finally, the in vitro chemical antioxidant capacities were evaluated using both the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assays.

RESULTS E DISCUSSION

We did not found any signs that BHT uncouples oxidative phosphorylation nor that it inhibits the respiratory chain in the perfused liver in a significant way, although these effects seem to be clear in isolated mitochondria. Contrary to what is expected from uncouplers, BHT did not significantly stimulate oxygen uptake nor had an inhibitory action on glucose synthesis from lactate. Actually, there was no inhibition of glucose synthesis or oxygen consumption in the concentration range up to 200 μM. Only at the BHT concentration of 500 μM small increments in both parameters were found. Pyruvate production presented an increasing tendency at 50 μM, followed by a peak increment at 100 μM and a subsequent return to the basal levels. Experiments were also done with very low concentrations of BHT, namely 1 μM (10^{-6} M), by virtue of the report that BHT might uncouple phosphorylation at very low concentrations (10^{-9} to 10^{-6} M). However, no modifications of oxygen uptake, glucose production and pyruvate production were found at low concentrations. Transformation of fructose into glucose was also not affected by BHT. Only lactate production was slightly increased at the concentration of 100 μM. However, in the passage from 100 to 200 μM BHT the production of lactate returned to the basal rates. Besides this, there was also a small increase in oxygen uptake, significant only at the concentration of 200 μM, and an increasing tendency for pyruvate production in parallel with the increase in lactate production. Altogether, this results demonstrate lack of an inhibitory action of BHT on glucose synthesis, suggesting a very weak interaction of the compound with the respiratory chain of the mitochondria inside the liver cells. The uncoupling effect of BHT in isolated mitochondria was confirmed, but only at concentrations above 10 μM. Uncoupling at lower concentrations, 10^{-9} to 10^{-6} M, could not be confirmed. BHT, however, increased ROS production in isolated mitochondria, starting at the concentration of 10^{-8} M. This is the opposite of what can be expected from a compound with proven ex vivo antioxidant action. Despite of its prooxidant action in mitochondria, BHT was an effective antioxidant in the ABTS and DPPH assays. Additionally, all 13 other antioxidants tested were capable of acting as free radical scavengers on both chemical assays, although to different degrees and with discrepancies between the two methodologies. At least four of them considerably enhanced the net ROS production in mitochondria: BHT, BHA (butylated

hydroxyanisole), gallic acid and ascorbic acid. As already mentioned, BHT increased considerably ROS production starting at the concentration of 10^{-8} M and had a maximal stimulation of 59% at 10^{-3} M. BHA also started to increase the net ROS production at the concentration of 10^{-8} M, and the final increment at 10^{-3} M (49%) was less pronounced than that caused by BHT. Ascorbic acid increased slightly the ROS production at the lowest concentrations, but from 10^{-5} until 10^{-3} M progressive increments occurred, reaching the final value of 44% stimulation. Gallic acid already showed maximal stimulation (62%) at the lowest concentration examined in the present work, namely 10^{-9} M. Therefore, a good correlation between the antioxidant activity in the chemical systems and the mitochondria cannot be expected. Some disparities are very pronounced. This is the case, for example, of ascorbic acid, which was the worst $ABTS^{+}$ free radical scavenger, the best DPPH free radical scavenger and a net stimulator of ROS production in the mitochondria. The reasons why very effective free radical scavengers in chemical systems, such as gallic acid and several others, present either a poor performance in the mitochondria or are even capable of stimulating ROS production, is possibly linked to the complexity of the biological structures which allow a great number of interactions of various kinds. Another group of efficient free radical scavengers in artificial systems were poorly active in the mitochondria, as for example propyl gallate and quercetin. The four most effective antioxidants in the mitochondria, with IC_{50}'s smaller than 10 μM were: resveratrol < chlorogenic acid < epicatechin < coumaric acid. It is already known that several of the compounds tested in the present work can exert a dual role, i.e., they can act as antioxidants under certain situations and in specific places or as prooxidants under distinct conditions. To act as an antioxidant or as a prooxidant, thus, seems to depend not only on the specific conditions in a given biological system but also on the biological system with which the compound is interacting.

CONCLUSIONS

Prooxidant activity of BHT in mitochondria is the opposite what can be expected for a compound that is generally known as an antioxidant. One cannot exclude the possibility that, in mitochondria, *in vitro* or even *in vivo*, stimulation of ROS production rather than uncoupling could be the most significant effect of BHT, since the first occurred at low concentrations, while the second only ocurred at relatively high concentrations that, apparently, cannot be reached when this substance is given to intact cells. On the other hand, the results also reinforce the notion that demonstration of free radical scavenging activity for a given compound in chemical *in vitro* assays is no guarantee that it will act as an antioxidant in the mitochondria.

Key-words: antioxidants, butylated hydroxytoluene, liver metabolism.

RESUMO GERAL

INTRODUÇÃO

O hidroxitolueno butilado (BHT) é amplamente utilizado como antioxidante pela indústria alimentícia, cosmética, farmacêutica e em produtos derivados do petróleo. É também comercializado em cápsulas como suplemento alimentar para a saúde. Como antioxidante, inibe a peroxidação lipídica, efeito que também pode exercer em sistemas biológicos. Sabe-se que a ingestão de substâncias capazes de eliminar radicais livres (antioxidantes) pode, pelo menos em parte, evitar os efeitos deletérios das espécies reativas de oxigênio (ROS). A geração dessas espécies nas mitocôndrias é muito importante, podendo contribuir significativamente para danos em organelas e em várias patologias. Sendo assim, bons eliminadores de radicais livres devem estar ativos na menor concentração possível, permitindo a ingestão de doses mais baixas possíveis. A capacidade dos antioxidantes na eliminação dos radicais livres é geralmente avaliada utilizando ensaios químicos, como os métodos baseados na neutralização dos radicais gerados pelos compostos 1,1-difenil-2-picril-hidrazila (DPPH) e ácido 2,2'-azino-bis-3-etilbenzotiazolino-6-sulfônico (ABTS). Não existe garantia de que uma determinada substância ativa na eliminação de radicais livres num sistema artificial também seja capaz de eliminar com a mesma eficiência as espécies reativas de oxigênio produzidas pelas mitocôndrias. Em adição aos seus efeitos como antioxidante, potenciais efeitos adversos associados ao uso do BHT já foram relatados. Por exemplo, existem relatos de que o BHT é capaz solubilizar membranas lisossomais e desorganizar a estrutura das mitocôndrias, o que pode sugerir que este composto interfere com o metabolismo celular. De fato, estudos demonstram que o BHT interfere com o funcionamento mitocondrial por dois mecanismos distintos: desacoplamento entre o transporte de elétrons e a fosforilação oxidativa e inibição do complexo I da cadeia transportadora de elétrons. Estes dados sugerem, em teoria, que o BHT deve estimular ou inibir a captação de oxigênio, estimular as vias catabólicas e inibir as vias anabólicas. Tais ações além de outras, têm sido claramente demonstradas com vários desacopladores e inibidores respiratórios.

OBJETIVOS

Com base no exposto, o presente trabalho possui dois objetivos distintos: 1) realizar um estudo sistemático dos efeitos metabólicos do BHT no fígado de rato, utilizando o fígado em perfusão isolada e também mitocôndrias isoladas e 2) comparar a atividade sequestradora de radicais livres do BHT e de vários outros antioxidantes bem conhecidos em sistemas químicos com aquela exercida em mitocôndrias isoladas. Em relação a este último aspecto, este estudo deverá informar sobre o real potencial antioxidante do BHT e de diversos outros antioxidantes num sistema biológico (geração de ROS em mitocôndrias).

MATERIAL E MÉTODOS

Nos experimentos foram utilizados ratos machos Wistar, pesando 200- 280g. Todos os experimentos foram realizados de acordo com as diretrizes éticas aceitas mundialmente para experimentação em animais e foram previamente aprovadas pelo Comitê de Ética em Experimentação Animal da Universidade de Maringá (protocolo nº 9120290915). Para os experimentos de perfusão do fígado isolado de rato, foi utilizado um sistema de perfusão não-recirculante, livre de hemoglobina

(tampão Krebs/Henseleit- bicarbonato, pH 7,4, saturado com O_2:CO_2 na proporção 95:5). Ratos em jejum de 18 horas foram utilizados. O BHT foi dissolvido no líquido de perfusão e infundido em concentrações de 1 a 500 μM. Lactato e frutose foram utilizados como substratos neoglicogênicos e, a partir das amostras do perfusado, foram feitos ensaios enzimáticos para determinação da concentração de glicose, lactato e piruvato. O consumo de oxigênio foi monitorado continuamente por um eletrodo de platina. Dois ensaios distintos foram realizados para avaliar os efeitos do BHT sobre a atividade respiratória de mitocôndrias. Os primeiros ensaios foram aqueles clássicos utilizando mitocôndrias intactas e fosforilantes, onde a velocidade do consumo de oxigênio na ausência (estado IV) ou presença (estado III) de ADP foi avaliada, assim como o controle respiratório (RC). Nestes experimentos, succinato foi utilizado como substrato e o BHT foi utilizado numa faixa de concentração de 10 a 100 μM. Além destes experimentos, foram realizados ensaios com mitocôndrias não-fosforilantes, nos quais a oligomicina (inibidor clássico da ATP sintase) foi utilizada. Succinato foi também utilizado como substrato e o BHT testado numa faixa de concentração entre $2x10^{-9}$ a 10^{-4} M. Além disso, foi avaliado o efeito do BHT e de outros 13 antioxidantes diferentes sobre a produção de ROS em mitocôndrias intactas. A produção de ROS foi estimada através da medição do aumento de fluorescência linear (504 nm para excitação e 529 nm para emissão) devido à formação de 2'-7'-diclorofluoresceína (DCF) via oxidação por H_2O_2 na presença de peroxidase de raiz forte, utilizando succinato como substrato. Por fim, a capacidade antioxidante química *in vitro* do BHT e destes 13 antioxidantes foi avaliada utilizando os ensaios do ABTS e do DPPH.

RESULTADOS E DISCUSSÃO

Não foram encontrados sinais de que o BHT desacopla a fosforilação oxidativa no fígado perfundido de forma significativa, embora estes efeitos pareçam estar claros em mitocôndrias isoladas. Contrariamente ao que se espera de um desacoplador, o BHT não estimulou o consumo de oxigênio nem inibiu a neoglicogênese a partir do lactato. Isso porque na faixa de concentração até 200 μM não houve inibição da produção de glicose ou do consumo de oxigênio. Apenas nas concentrações de 500 μM pequenos aumentos em ambos os parâmetros foram encontrados. A produção de piruvato apresentou uma tendência a aumento na concentração de 50 μM, seguido por um pico com 100 μM, com posterior retorno aos níveis basais. Em virtude de relatos que o BHT poderia desacoplar a fosforilação em concentrações muito baixas (10^{-9} to 10^{-6} M), também foram feitos experimentos na concentração de 1 μM (10^{-6} M), porém não foram observadas modificações no consumo de oxigênio, produção de glicose e piruvato. A transformação da frutose em glicose também não foi afetada pelo BHT. Apenas na concentração de 100 μM a produção de lactato a partir de frutose foi aumentada para um grau apreciável. Porém na passagem de 100 a 200 μM a produção de lactato retornou às taxas basais. Paralelamente ao aumento na produção de lactato, houve um pequeno aumento no consumo de oxigênio apenas na concentração de 200 μM e uma tendência crescente na produção de piruvato. Estes resultados demonstram, portanto, a ausência de uma ação inibitória do BHT sobre a síntese de glicose, o que sugere uma interação muito fraca do composto com a cadeia respiratória das mitocôndrias dentro das células hepáticas. O efeito desacoplador do BHT em mitocôndrias isoladas foi confirmado, mas apenas em concentrações acima de 10 μM. Desacoplamento em concentrações mais baixas, 10^{-9} a 10^{-6} M, não pôde ser confirmado. No entanto, o BHT aumentou a produção de ROS em mitocôndrias isoladas, começando na concentração de 10^{-8} M, ou seja, houve uma estimulação da produção de ROS ao invés de ação antioxidante, como seria esperado. Embora

tenha estimulado a produção de ROS em mitocôndrias, o BHT atuou como um antioxidante eficaz nos ensaios do ABTS e do DPPH. Adicionalmente, todos os outros 13 antioxidantes investigados foram capazes de atuar como sequestrantes de radicais livres nos ensaios químicos, embora esta ação tenha ocorrido em diferentes graus e com discrepâncias entre as duas metodologias. Todavia, quatro deles estimularam claramente a produção de ROS em mitocôndrias de fígado de rato: BHT, BHA (hidroxianisol butilado), ácido ascórbico e ácido gálico. Como já relatado acima, o BHT aumentou consideravelmente a produção de ROS a partir da concentração de 10^{-8} M, atingindo 59% de estimulação na concentração de 10^{-3} M. O BHA também começou a aumentar a produção de ROS na concentração de 10^{-8} M e o estímulo final em 10^{-3} M (49%) foi menos acentuado do que pelo BHT. O ácido ascórbico aumentou ligeiramente a produção de ROS, atingindo o valor final de 44% na concentração de 10^{-3} M. O ácido gálico por sua vez, apresentou estímulo máximo (62%) na concentração mais baixa examinada no presente trabalho, 10^{-9} M. Assim, não se pode esperar uma boa correlação entre a atividade antioxidante nos sistemas químicos e nas mitocôndrias. Algumas disparidades foram muito pronunciadas. Este é o caso, por exemplo, do ácido ascórbico, que foi o pior removedor dos radicais livres do $ABTS^{+}$, o melhor depurador de radicais livres do DPPH e um estimulador líquido da produção de ROS nas mitocôndrias. Adicionalmente, as razões pelas quais os sequestradores de radicais livres muito eficazes, como o ácido gálico e vários outros, apresentam um fraco desempenho nas mitocôndrias ou são capazes de estimular a produção de ROS, possivelmente estão ligados à complexidade das estruturas biológicas que permitem um grande número de interações de vários tipos. Outros antioxidantes que se mostraram eficientes nos sistemas artificiais foram pouco ativos nas mitocôndrias, como por exemplo o propil galato e a quercetina. Os melhores sequestradores de radicais livres nas mitocôndrias, com valores de IC_{50} menores do que 10 μM, foram: resveratrol < ácido clorogênico < epicatequina < ácido cumárico. Sabe-se que vários dos compostos testados no presente trabalho podem exercer um duplo papel, isto é, podem atuar como antioxidantes em determinadas situações e em locais específicos ou como prooxidantes em condições distintas. Agir como um antioxidante ou como um prooxidante, assim, parece depender não apenas das condições específicas de um determinado sistema biológico, mas também do sistema biológico com o qual o composto está interagindo.

CONCLUSÕES

A atividade prooxidante do BHT nas mitocôndrias é o oposto do que se pode esperar de um composto tido em geral como antioxidante. Não se pode excluir a possibilidade de que, nas mitocôndrias, o estímulo da produção de ROS em vez do desacoplamento possa ser o efeito mais significativo do BHT. Isto porque ele ocorre em baixas concentrações, enquanto que o efeito desacoplador só ocorre em concentrações relativamente altas que, aparentemente, não são atingidas quando composto é fornecido às células intatas. Por outro lado, a comparação da capacidade sequestrante de radicais livres em sistemas químicos com aquela encontrada em mitocôndrias reforça a ideia de que a demonstração da atividade de eliminação de radicais livres em sistemas químicos para um determinado composto não é garantia de que ele atuará como um antioxidante nas mitocôndrias.

Palavras chaves: antioxidantes, hidroxitolueno butilado, metabolismo hepático.

Artigo 1

A reappraisal of the proposed metabolic and antioxidant actions of butylated hydroxytoluene (BHT) in the liver

Lorena dos Santos Castro, Lívia Bracht, Jurandir Fernando Comar, Rosane Marina Peralta and Adelar Bracht
University of Maringá, Department of Biochemistry, 87020900 Maringá, Brazil

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Corresponding author

Adelar Bracht
Department of Biochemistry
University of Maringá
87020900 Maringá, Brazil
Email: adelarbracht@bol.com.br

ABSTRACT

Butylated hydroxytoluene (BHT) was investigated for its metabolic actions in the perfused rat liver. Contrary to what is expected from an uncoupler, BHT up to 500 μM did not stimulate oxygen uptake nor did it inhibit gluconeogenesis from lactate. Transformation of fructose into glucose was also not affected by BHT; only lactate production was slightly increased at the concentration of 100 μM. The uncoupling effect of BHT in isolated mitochondria was confirmed, but only at concentrations above 10 μM; uncoupling at lower concentrations, 10^{-9} to 10^{-6} M, could not be confirmed. BHT, however, increased ROS production in isolated mitochondria, starting at the concentration of 10^{-8} M. This is the opposite of what can be expected from a compound with proven ex vivo antioxidant action. One cannot exclude the possibility that, in mitochondria, stimulation of ROS production rather than uncoupling could be the most significant effect of BHT.

INTRODUCTION

Butylated hydroxytoluene (BHT) is amply utilized as antioxidant in industrially processed foods, cosmetics, pharmaceutical and petrochemical products.[1] Its chemical structure is shown as an inset in Figure 1. It is a highly hydrophobic compound which presents high potential of interacting with biological membranes, enzymes linked to membranes and hydrophobic domains of proteins in general. As antioxidant, BHT inhibits lipid peroxidation, an effect that it also exerts in biological systems.[2] It was also shown, however, that BHT labilizes lisosomal membranes and disorganizes the mitochondrial structure.[3] This potential toxicity of BHT does not avoid its commercialization in the form of capsules as a food and health supplement. It has also been reported that the compound exerts antiviral effects, particularly against the herpes virus family.[4,5] There is also some popular literature about the latter effect.[6]

The most obvious interaction of BHT with the liver is biotransformation, since this organ uses to be the most important site of the transformation of xenobiotics. The biotransformation of BHT in the liver is characterized by hydroxylations.[7] Hydroxylation of the methyl group in position 4 culminates with the formation of a carboxyl group, an event that facilitates renal excretion. The hydroxylated and carboxylated products are probably inert, but another biotransformation product, namely the BHT-quinone methide, is highly electrophilic and able to conjugate with reduced glutathione (GSH) as well as with macromolecules such as proteins.[8,9] One of the most prominent toxic effects of BHT is tissue necrosis, which results from the reaction of the BHT-quinone methide with macromolecules.[10]

With respect to energy metabolism there are at least two points of view on how the compound impairs the mitochondrial energy transduction. The first point of view regards BHT as a classical uncoupler or inhibitor of electron flow. In this view BHT impairs mitochondrial energy transduction by a combination of uncoupling and inhibition of complex I at concentrations in the range between 50 and 750 μM.[11,12] The pertinent experiments were done using phosphorylating mitochondria with respiratory rate measurements in the presence and absence of ADP or submitochondrial particles.[11] The second view was derived from experiments in which predominantly non-phosphorylating mitochondria were used.[13] In the latter approach the authors found oxygen uptake stimulation by BHT in the range from 10^{-9} to 10^{-6} M. If this represents uncoupling, BHT would possess a dynamic or ample uncoupling range. This could be a useful property in contrast to other uncouplers

possessing a narrower range in that it would diminish the risks of overdosage when using uncouplers for weight loss and other health promoting purposes.[14,15]

Irrespective of the range in which BHT acts as an uncoupler or respiratory chain inhibitor, it has not yet been unequivocally demonstrated that this compound really acts as such in intact cell systems. In theory BHT should either stimulate or inhibit oxygen uptake, stimulate catabolic pathways and inhibit anabolic pathways.[16,17,18] Such actions, beside others, have been clearly demonstrated to occur with several uncouplers and respiratory inhibitors. The purpose of the present work was to examine this question in the isolated perfused rat liver, a system in which the microcirculation is preserved in addition to the cell integrity. The results should bring additional information about the interactions of BHT with the liver and perhaps shed a new light on what can be expected from this compound in terms of physiologic effects.

MATERIALS AND METHODS

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringa. Bytulated hydroxytoluene, enzymes and coenzymes used in the enzymatic assays were purchased from Sigma-Aldrich Co (St. Louis, USA). All other chemicals were from the best available grade (98–99.8% purity).

Animals

Male Wistar rats weighing 200–280 g were used in all experiments. Animals were fed ad libitum with a standard laboratory diet (Nuvilab®, Colombo, Brazil) and maintained on a regulated light–dark cycle. In accordance with the protocol, rats were used fed or starved for 18 h prior to the experiments. For preparing the liver for perfusion the rats were anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg). The criterion of anesthesia was the lack of body or limb movement in response to a standardized tail clamping stimulus. All experiments were done in accordance with the worldwide accepted ethical guidelines for animal experimentation and were previously approved by the Ethics Committee of Animal Experimentatin of the University of Maringá (protocol n. 9120290915).

Liver perfusion

Hemoglobin-free, non-recirculating perfusion was performed.[19,20] After cannulation of the portal and cava veins the liver was positioned in a plexiglass chamber. The constant flow was provided by a peristaltic pump (Minipuls 3, Gilson, France) and was adjusted between 30 and 32 mL/min, depending on the liver weight. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 250 mg% bovine-serum albumin, saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment (37 °C). The composition of the Krebs/Henseleit-bicarbonate buffer is the following: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 2.5 mM $CaCl_2$. The perfusion fluid enters the liver via a cannula inserted into the portal vein and leaves the organ via a cannula inserted into the cava vein.[19,20] Samples of the effluent perfusion fluid were collected and analyzed for their metabolite contents. Substrates and BHT were added to the perfusion fluid according to the experimental protocols. Due to

its low water solubility, BHT was added to the perfusion fluid as a dimethylsulfoxide solution to achieve the desired final concentration. BHT is highly soluble in DMSO and the latter is frequently the solvent of choice in biological experiments.[11,13,21] Furthermore, it is already amply documented that dimethylsulfoxide does not significantly affect liver metabolism, at least not when infused at rates up to 32 µL/min,[22] a limit that was never surpassed in the present work. On the other hand, in aqueous buffers, BHT is stable to light and at pH values under 9.0.[23]

Metabolite assay

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their metabolite contents. The following compounds were assayed by means of standard enzymatic procedures: glucose, lactate and pyruvate.[24] The oxygen concentration in the outflowing perfusate was monitored continuously employing a Teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate.[20] Metabolic rates were calculated from input–output differences and the total flow rates and were referred to the wet weight of the liver.

Mitochondria isolation and measurement of respiratory activity

Fed rats were decapitated and their livers removed immediately and placed in ice-cold buffer containing 200 mM mannitol, 75 mM sucrose, 0.2 mM ethylene glycol tetraacetic acid (EGTA), 2 mM tris (hydroxymethyl)amino-methane (Tris–HCl), pH 7.4 and 50 mg% bovine serum albumin. The tissue was minced, washed with the buffer and homogenized in the same medium by means of a van Potter homogenizer for lysing the cells. After homogenization, the mitochondria were isolated by differential centrifugation and suspended in the same medium, which was kept at 0–4 °C.[25,26]

Oxygen uptake by isolated mitochondria was measured polarographically using a teflon-shielded platinum electrode.[26,27] For measuring oxygen uptake by phosphorylating mitochondria the organelles were incubated in the closed oxygraph chamber at 37 °C in a medium (2.0 mL) containing 0.25 M mannitol, 5 mM sodium diphosphate, 10 mM KCl, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 25 mg% fatty acid free bovine serum albumin and 10 mM Tris–HCl (pH 7.4). Succinate (10 mM) was used as the substrate. ADP, for a final concentration of 125 µM, was added at appropriate times. BHT was added as DMSO solutions with

different concentrations to ensure a constant amount of solvent. Controls were run to exclude solvent effects. The final BHT concentrations were in the range between 10 and 100 μM. Rates of oxygen uptake were computed from the slopes of the recorder tracings and expressed as nmol min^{-1} (mg protein)$^{-1}$. The respiratory control ratio (RC) was calculated as the ratio between the mean respiration rate during ADP phosphorylation (state III) and the respiration rate after ADP exhaustion (state IV).[28]

For measuring oxygen uptake by non-phosphorylating mitochondria,[13] freshly prepared intact mitochondria at a concentration of 0.5 mg protein/mL were incubated in the oxygraph chamber at 37 °C. The medium (2 mL) contained 120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes (pH 7.2) and 1 mM EGTA, 5 μM rotenone and 1 μg/ml oligomycin. Respiration was initiated by adding succinate to a final concentration of 4 mM. BHT, for final concentrations between 2×10^{-9} to 10^{-4} M, was added as DMSO solutions with different concentrations to ensure a constant amount of solvent (20 μL).

Protein content of the mitochondrial suspensions was measured using the folin-phenol reagent and bovine serum albumin as standard.[29]

Mitochondrial reactive oxygen species (ROS) production

The rate of mitochondrial ROS production (real time ROS production), basically H_2O_2, was estimated by measuring the linear fluorescence increase (504 nm for excitation and 529 nm for emission) due to 2'-7'-dichlorofluorescein (DCF) formation from the reduced form of 2'-7'-dichlorofluorescein (DCFH) via oxidation by H_2O_2 in the presence of horseradish peroxidase.[30,31] Intact mitochondria (0.5 mg) were incubated in 2 mL of a medium containing 250 mM mannitol, 1.36 μM 2'-7'-dichlorofluorescein acetate (DCFA-DA), 10 mM HEPES buffer (pH 7.2), 10 μM rotenone, 10 mM succinate as respiratory substrate and BHT in the range from 10^{-9} to 10^{-3} M or chlorogenic acid in the range from 10^{-8} to 10^{-3} M. The latter two were added as DMSO solutions. The acetate group of 2'-7'-dichlorofluorescein acetate is removed by endogenous esterases producing the reduced DCFH. Fluorescence due to 2'-7'-dichlorofluorescein (DCF) formation in consequence of ROS production was recorded during 10 min under agitation. The results were expressed as nmol·min^{-1}·(mg·protein)$^{-1}$. Appropriate controls were run in order to exclude the possibility of an interference of BHT with the fluorescence of 2'-7'-dichloro-fluorescein (DCF).

In vitro chemical antioxidant capacity

The in vitro chemical antioxidant capacity of BHT was evaluated using both the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assays.[32,33]

The cationic ABTS form was generated by incubating 3.7 mM ABTS with 1.3 mM potassium persulfate in the dark for 12 hours. After this time the incubation solution was diluted 60-fold with methanol. The assay system, 300 µL, was composed of 285 µL of the methanolic cationic ABTS solution (61 µM) plus 15 µL of a DMSO solution containing the desired BHT concentration. After one hour the absorbance decrease was measured at 734 nm.

The DPPH assay system (300 µL) was composed of 285 µL ethanolic 10.5 mM DPPH to which samples of 15 µL containing various concentrations of BHT in DMSO were added. After one hour in the dark the absorbance at 515 nm was measured.

Statistical analysis

The error parameters presented in the graphs are standard errors of the mean. Statistical analysis was done by means of the GraphPadPrism software (version5.0). Variance analysis was done with post-hoc testing according to Student-Newman-Keuls ($p \leq 0.05$).

RESULTS

Lactate gluconeogenesis

Gluconeogenesis from lactate in the liver is strictly dependent on an efficient energy transduction in the mitochondria. As an inhibitor of energy metabolism, BHT should affect this biosynthetic route as do other inhibitors.[12,16-18,22] Oxygen uptake is expected to be increased by pure uncoupling, decreased by a significant inhibition of the respiratory chain or remain more or less constant in the case of a balance between both phenomena. Irrespective of the net effect on oxygen uptake, however, the result is always inhibition of ADP phosphorylation, which inevitably leads to decreased gluconeogenesis. The experiments that were done with 2 mM lactate are illustrated by both panels of Figure 1. Livers from 18 hours fasted rats were perfused in order to ensure low glycogen levels. Under such conditions the rate of glucose output reflects mainly the rate of gluconeogenesis. Figure 1A shows the time course of the experiment that was done with 100 µM BHT in the portal perfusate. Figure 1A also illustrates the experimental protocol which was the same for all BHT concentrations. Before the infusion of lactate the rates of glucose and pyruvate output were minimal due to the lack of substrates, but oxygen uptake is relatively high because the liver respires at the expense of endogenous fatty acids (Figure 1A).[17,20] Upon the infusion of lactate all variables increased until a new steady-state was reached, which is characterized by high rates of glucose and pyruvate productions and a substantially increased rate of oxygen uptake. The introduction of 100 µM BHT did not cause significant modifications in oxygen uptake or glucose production. There was a tendency of increasing oxygen uptakte, but without statistical significance. The only significant effect of 100 µM BHT was a clear increase in pyruvate production of approximately 25%. Experiments were also done with very low concentrations of BHT, namely 1 µM, by virtue of the report that BHT might uncouple phosphorylation at very low concentrations.[13] The results were all negative, i.e., no modifications of oxygen uptake, glucose production and pyruvate production were found at low concentrations (experiments not shown).

In Figure 1B the results obtained in all the experiments in which BHT was infused in the range from 1 to 500 µM are summarized. The rates measured at the end of each BHT infusion period of 20 minutes were represented against the portal BHT concentration. Rates in the absence of BHT were also represented (zero BHT). There was no inhibition of glucose output and oxygen consumption in the

concentration range up to 200 μM. Only at the BHT concentration of 500 μM small increments in both parameters were found. Pyruvate production presented an increasing tendency at 50 μM, followed by a peak increment at 100 μM (see Figure 1A) and a subsequent return to the basal levels. It should be stressed that 500 μM is a very high concentration in physiological terms and it was also the maximal concentration that could be employed without precipitation of BHT in the perfusion fluid.

Fructose metabolism

In the liver fructose undergoes both an anabolic and energy-dependent conversion into glucose e a catabolic breakdown into lactate and pyruvate (fructolysis). For this reason the fructose metabolism is also affected by the energy status of the mitochondria. In the experiments that were done with fructose the experimental protocol was similar to that already employed when investigating lactate metabolism. The results are illustrated by Figure 2. The infusion of fructose resulted, as expected, in pronounced increases in glucose, lactate and pyruvate production and increases in oxygen consumption (Figure 2A). The introduction of 100 μM BHT, on the other hand, did not cause pronounced changes in the new steady-state rates. Actually, only lactate production was increased to an appreciable degree when 100 μM BHT was infused, a phenomenon that is more evident in Figure 2B which shows the concentration dependence of the modifications caused by BHT. In the passage from 100 to 200 μM BHT the production of lactate returned to the basal rates. Besides this, there was also a small increase in oxygen uptake, significant only at the concentration of 200 μM, and an increasing tendency for pyruvate production in parallel with the increase in lactate production. Glucose production was not affected by BHT in the range up to 500 μM.

Mitochondrial respiration

Considering previous reports about the action of BHT on the respiratory activity of isolated rat liver mitochondria,[11,13] and the minimal responses of the perfused liver described above, it seems reasonable to verify if some of these observations can be reproduced under our conditions. Figure 3 shows an attempt of reproducing the experiments that were done in the past with phosphorylating rat liver mitochondria using the classical approach in which the addition of the substrate to the medium

containing respiring mitochondria is followed by the addition of ADP.[11] The substrate was succinate and the BHT concentration was in the range up to 100 μM. Basal substrate respiration was increased by BHT with a maximum in the range between 50 and 70 μM, but with an inhibitory tendency when the concentration was increased to 100 μM. State III respiration diminished with increasing BHT concentrations, but a clear definition of the inhibitory action began at the concentration of 50 μM. State IV respiration, finally, tended to increase to values equal to state III respiration. The equality was reached at the concentration of 50 μM. The consequence was that the respiratory control ratio was progressively diminished when the BHT concentrations were increased, tending to unity. All these observations reproduce a previous report, indicating an uncoupling effect of BHT when in contact with isolated rat liver mitochondria, but in the range between 10 and 100 μM.[11]

The increase in mitochondrial respiration caused by very low BHT concentrations, observed in a previous work,[13] was not reproduced. The medium used in these experiments, whose results are shown in Figure 4, included oligomycin, an inhibitor of the ATP-synthase, thus preventing phosphorylation. Respiration was driven by succinate alone, because the medium also contained rotenone.[13] No significant stimulation of oxygen uptake was found in the range between 2×10^{-9} M and 5×10^{-5} M (50 μM). Almost complete inhibition of oxygen uptake occurred, on the other hand, in the passage from 50 μM to 100 μM. It is important to note that uncoupling by FCCP (carbonyl cyanide-*4*-(trifluoromethoxy)-phenylhydrazone) resulted in a clear and pronounced stimulation of oxygen uptake, showing that the respiratory chain of the mitochondria used in the experiments were perfectly able to respond to protonophores.

Mitochondrial ROS generation

Failure of increasing oxygen uptake in mitochondria under non-phophorylating conditions by very low BHT concentrations in the present work could be the consequence of the presence of ligand-proteins or other agents hindering the access of the compound to the mitochondrial structures. If a sequestration of BHT was occurring in the experiments shown in Figure 4, other effects that can be expected to be caused by relatively low concentrations of BHT should be equally prevented. The ROS scavenging activity of BHT occurs at relatively low concentrations in chemical systems, as revealed by Figure 5A. The compound

should equally be able to scavenge ROS produced in the mitochondria at low concentrations. For this purpose ROS production by mitochondria respiring at the expense of succinate was measured in the presence of several BHT concentrations in the range from 10^{-9} to 10^{-3} M. [30,31,34] Results are shown in Figure 5B. Surprisingly, BHT was indeed active, but not as a scanveger but rather as an enhancer of ROS production, starting at the concentration of 10^{-8} M. The curve was biphasic in that after a stabilization at 10^{-5} M a new increase was observed when the concentration was raised further. As a positive control, experiments were conducted in parallel with several antioxidants, including chlorogenic acid, for which the results were also plotted in Figure 5B. The action of chlorogenic acid as a ROS scavenger began at 10^{-8} M and was almost complete at concentrations around 10^{-4} M. It seems, thus, that BHT was available to the mitochondria in our incubations at very low concentrations, at least to the point of being able to contribute to the generation of reactive oxygen species.

DISCUSSION

We did not found evidence that BHT uncouples oxidative phosphorylation in the perfused liver neither did we found inhibition of electron flow, although these effects seem to be clear in isolated mitochondria. This observation is in sharp contrast with those made with several other highy lipophillic uncouplers, that act very quickly after introduction in the portal vein.[16-18,22,35] Differences in concentration dependence between the action on isolated mitochondria or isolated cells and the intact tissue are frequent phenomena. In a perfused organ the ratio extracellular volume to cell volume is smaller than unity whereas in incubations with isolated mitochondria or cells this ratio can reach orders of magnitude above unity. Furthermore, the continuous renovation of the extracellular fluid by the microcirculation prevents the accumulation of toxic products. Even so, the lack of an inhibitory action of BHT on glucose synthesis especially, denotes a very weak interaction of the compound with the respiratory chain of the mitochondria inside the liver cells. No permeation of the cell membrane is unlikely. In [^{14}C]BHT-treated rats considerable binding to intracellular macromolecules was found,[36] a clear indication that cell permeation must have occurred. Membrane permeation is in any case expected due to the lipophilic nature of BHT. Intense binding to intracellular molecules and structures, both reversible and irreversible,[36] on the other hand, could at least in part explain the poor action on the respiratory chain and energy metabolism in the perfused rat liver.

Contrary to a previous claim,[11] thus, impairment of energy metabolism in the liver seems not to be an important mechanism of BHT toxicity.[8,9,10] On the other hand, it seems also irrealistic to expect that BHT might exert beneficial effects as a mild uncoupler at low concentrations. It must be mentioned here at this respect that mild uncoupling has not been recommended only for weight loss purposes.[37] It has also been claimed, for example, that mild uncoupling increases longevity in mice a phenomenon that was associated with the improvement of several serological markers such as glucose, triglycerates and insulin levels.[15] This and other observations provide the basis for the search of uncouplers with dynamic range, which could prevent the dangers of overdosage. However, we did not find oxygen uptake stimulation at low concentrations (up to 1 μM) in the perfused liver and also not in non-phosphorylating mitochondria, the phenomenon on which the proposed action of BHT as an uncoupler with dynamic range was based.[13] The only component in our incubation system that was not present when compared to

the original observation[13] was nigericin, a H^+/K^+ antiporter.[38] However, if oxygen uptake stimulation by BHT at low concentrations depends on the presence of nigericin, the phenomenon can hardly have a physiological significance, as this agent is extraneous to mammalian cells.

What we have found in mitochondria at low concentrations of BHT was actually the opposite of what is normally expected from this compound, namely an antioxidant action.[1,2] That this action occurs at very low concentrations in intact mitochondria, starting at 10^{-8} M, can be an important detail because, as discussed above, it is highly probable that the concentrations of free (unbound) BHT in the cell are very low. One cannot exclude the possibility that, in mitochondria, in vitro or even in vivo, stimulation of ROS production rather than uncoupling could be the most significant effect of BHT.

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Legends to the figures

FIGURE 1 Effects of BHT on lactate gluconeogenesis and associated variables in the perfused rat liver. Livers from fasted rats were perfused as described in the Materials and Methods section. Lactate was infused during 60 min (10–70 min perfusion time). Samples of the effluent perfusion fluid were collected for the enzymatic metabolite assay. Oxygen consumption was monitored polarographically. Panel A: time course of the effects of 100 μM BHT, which was infused during 22 min (36–58 min perfusion time). Panel B: concentration dependence of the changes promoted by BHT after 20 min infusion (56 min perfusion time). Each datum point represents the mean of 3-4 liver perfusion experiments. Bars are standard errors of the mean. Asterisks in panel B indicate statistical difference relative to the absence of BHT in the perfusion fluid ($p \leq 0.05$).

FIGURE 2 Effects of BHT on fructose metabolism in the perfused rat liver. Livers from fasted rats were perfused as described in the Materials and Methods section. Fructose was infused during 60 min (10–70 min perfusion time). Samples of the effluent perfusion fluid were collected for the enzymatic metabolite assay. Oxygen consumption was monitored polarographically. Panel A: time course of the effects of 100 μM BHT, which was infused during 20 min (36–56 min perfusion time). Panel B: concentration dependence of the changes promoted by BHT after 20 min infusion (56 min perfusion time). Each datum point represents the mean of 3-4 liver perfusion experiments. Bars are standard errors of the mean. Asterisks in panel B indicate statistical difference relative to the absence of BHT in the perfusion fluid ($p \leq 0.05$).

FIGURE 3 Effects of BHT on the respiratory activity of isolated phosphorylating rat liver mitochondria. Freshly prepared intact mitochondria at a concentration of 1 mg protein/mL were incubated at 37 °C in a closed oxygraph chamber. The medium (2.0 mL) contained 250 mM mannitol, 10 mM KCl, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 25 mg% fatty acid free bovine serum albumin and 10 mM Tris–HCl (pH 7.4). Succinate (10 mM) was used as the

substrate. ADP, for a final concentration of 125 µM, was added at appropriate times. BHT was added as DMSO solutions with different concentrations to ensure a constant amount of solvent. Each datum point is the mean±SEM of 3 independent experiments. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Student-Newman–Keuls testing ($p \leq 0.05$).

FIGURE 4 Effects of BHT on the respiratory activity of non-phosphorylating isolated rat liver mitochondria. Freshly prepared intact mitochondria at a concentration of 0.5 mg protein/mL were incubated in a closed oxygraph chamber at 37 °C. The medium contained 120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes (pH 7.2) and 1 mM EGTA, 5 µM rotenone and 1 µg/ml oligomycin. Respiration was initiated by adding succinate to a final concentration of 4 mM. BHT was added as DMSO solutions with different concentrations to ensure a constant amount of solvent (20 µL). The FCCP (carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone) concentration in the corresponding control experiments was 0.3 µM. Each datum point is the mean±SEM of 3 independent experiments.

FIGURE 5 Free radical scavenging activity of BHT in chemical systems (A) and its effects on the mitochondrial generation of reactive oxygen species (B). The chemical free radical scavenging activities of BHT were estimated as its capacity of scavenging the DPPH radical (1,1-diphenyl-2-picryl-hydrazyl) and of reacting with the ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) radical cation. The results were expressed as changes in absorbance at 515 nm for the DPPH assay and 734 nm for the ABTS assay. The rate of mitochondrial ROS production by freshly isolated mitochondria was estimated by measuring the linear fluorescence increase (504 nm for excitation and 529 nm for emission) due to oxidized 2'-7'-dichlorofluorescein (DCF) formation from the reduced form (DCFH) via oxidation by H_2O_2 in the presence of horseradish peroxidase. More details are given in the Materials and Methods section. All values represent the mean of 3 determinations. Vertical bars represent standard errors of the mean (not visible when smaller than the symbol size). Statistical significance for the mitochondrial assays is indicated by asterisks (*), as computed by variance analysis with post hoc Student-Newman–Keuls testing ($p \leq 0.05$).

Figure 1

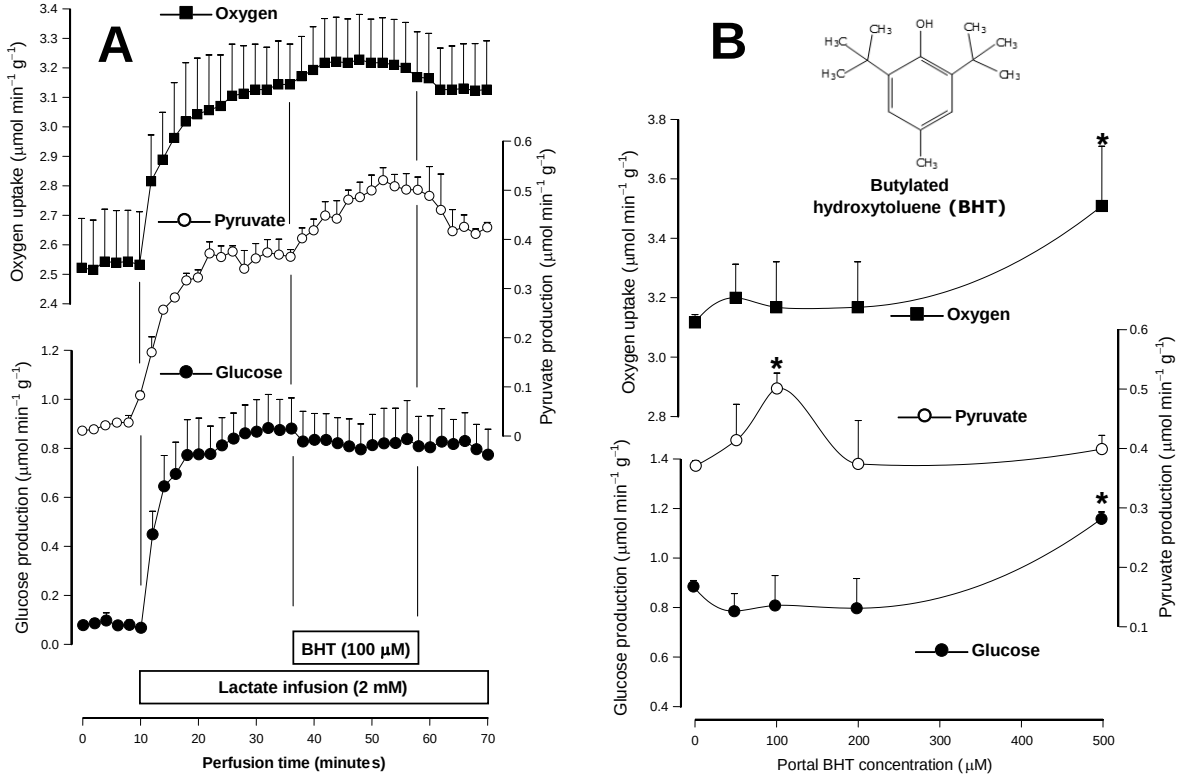

A
Oxygen
Pyruvate
Glucose
Oxygen uptake (μmol min⁻¹ g⁻¹)
Pyruvate production (μmol min⁻¹ g⁻¹)
Glucose production (μmol min⁻¹ g⁻¹)
BHT (100 μM)
Lactate infusion (2 mM)
Perfusion time (minutes)
B
Butylated hydroxytoluene (BHT)
Portal BHT concentration (μM)

Figure 2

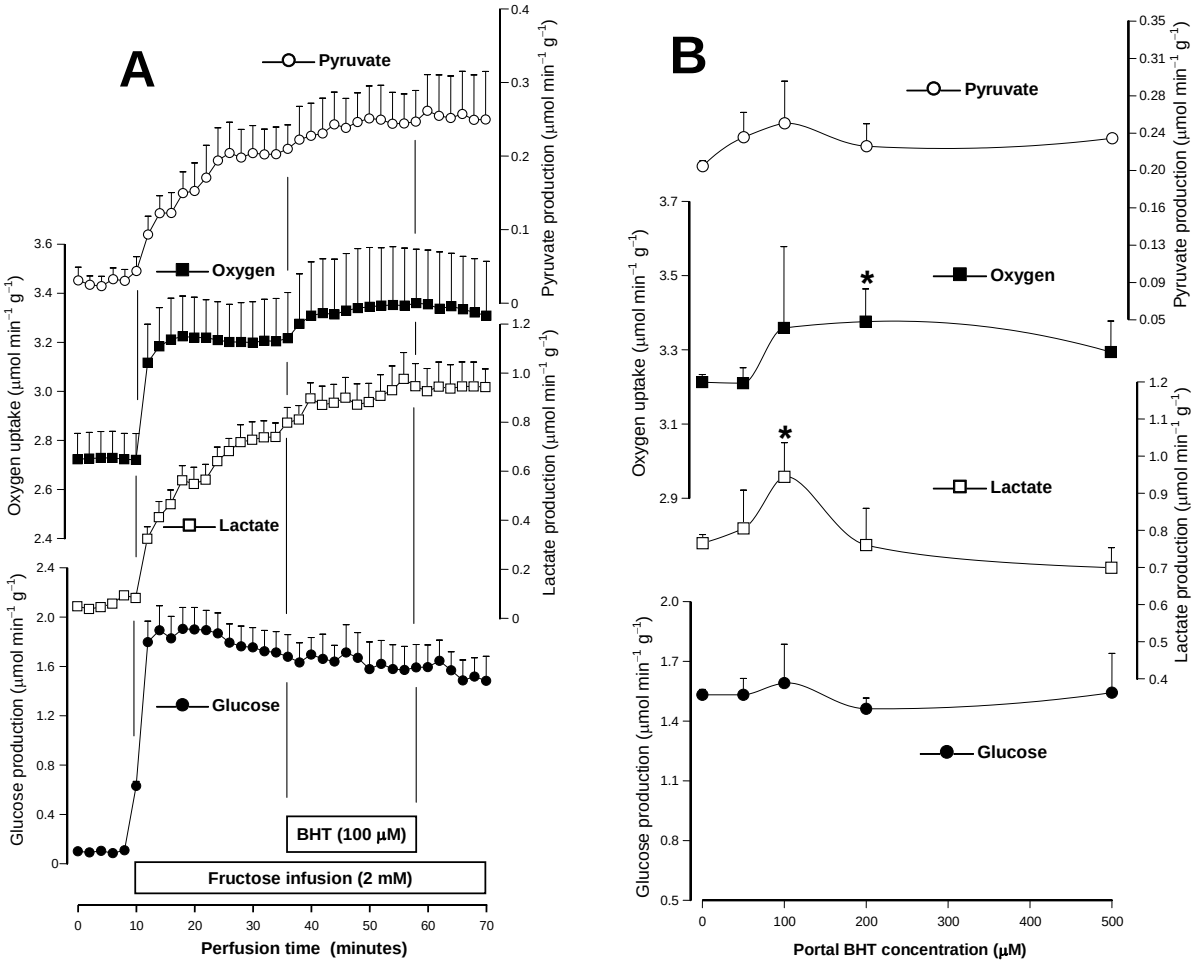

A
Pyruvate
Oxygen
Lactate
Glucose
Oxygen uptake (µmol min⁻¹ g⁻¹)
Pyruvate production (µmol min⁻¹ g⁻¹)
Lactate production (µmol min⁻¹ g⁻¹)
Glucose production (µmol min⁻¹ g⁻¹)
BHT (100 µM)
Fructose infusion (2 mM)
Perfusion time (minutes)
B
Pyruvate
Oxygen
Lactate
Glucose
*
*
Oxygen uptake (µmol min⁻¹ g⁻¹)
Pyruvate production (µmol min⁻¹ g⁻¹)
Lactate production (µmol min⁻¹ g⁻¹)
Glucose production (µmol min⁻¹ g⁻¹)
Portal BHT concentration (µM)

Figure 3

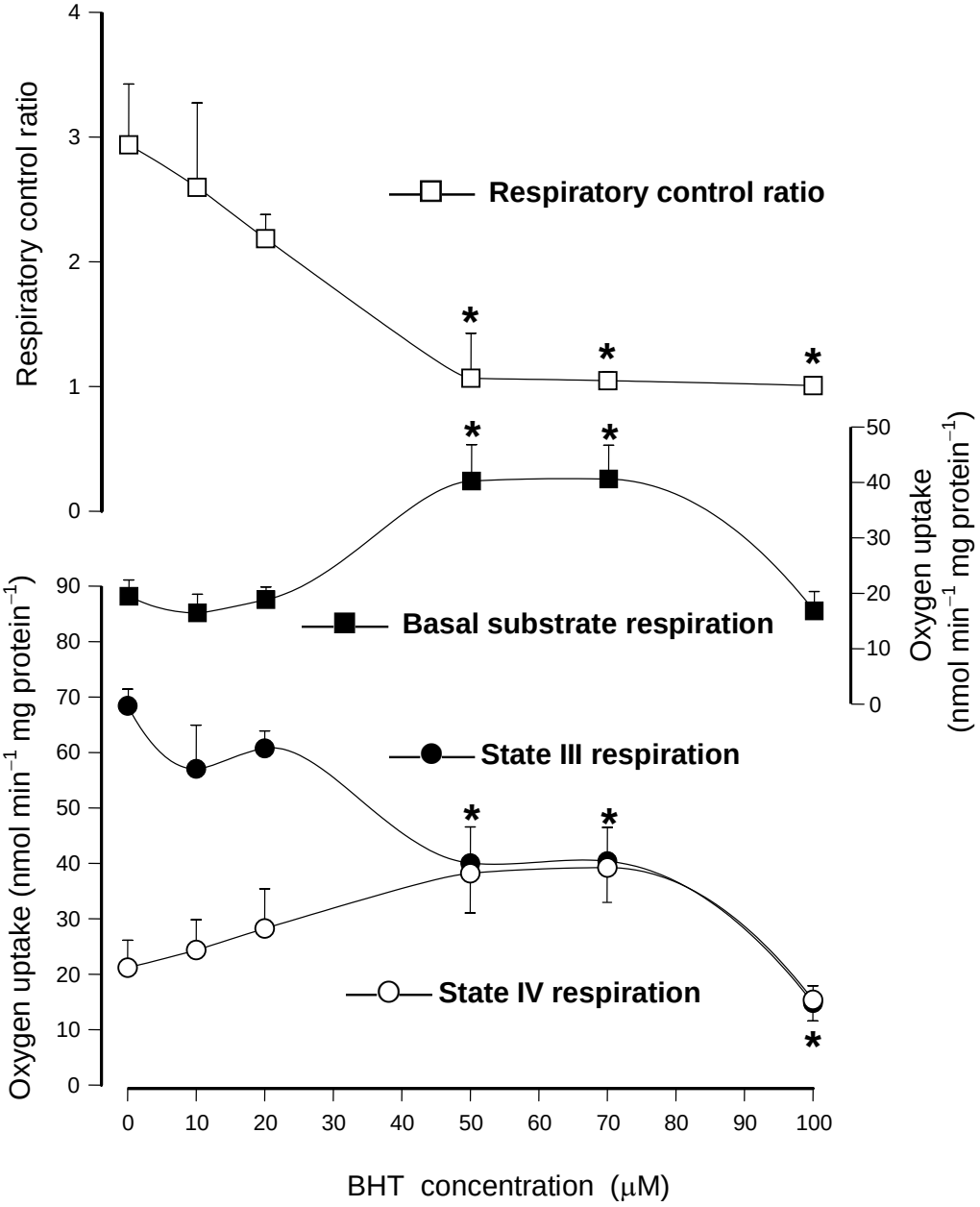


Figure 4

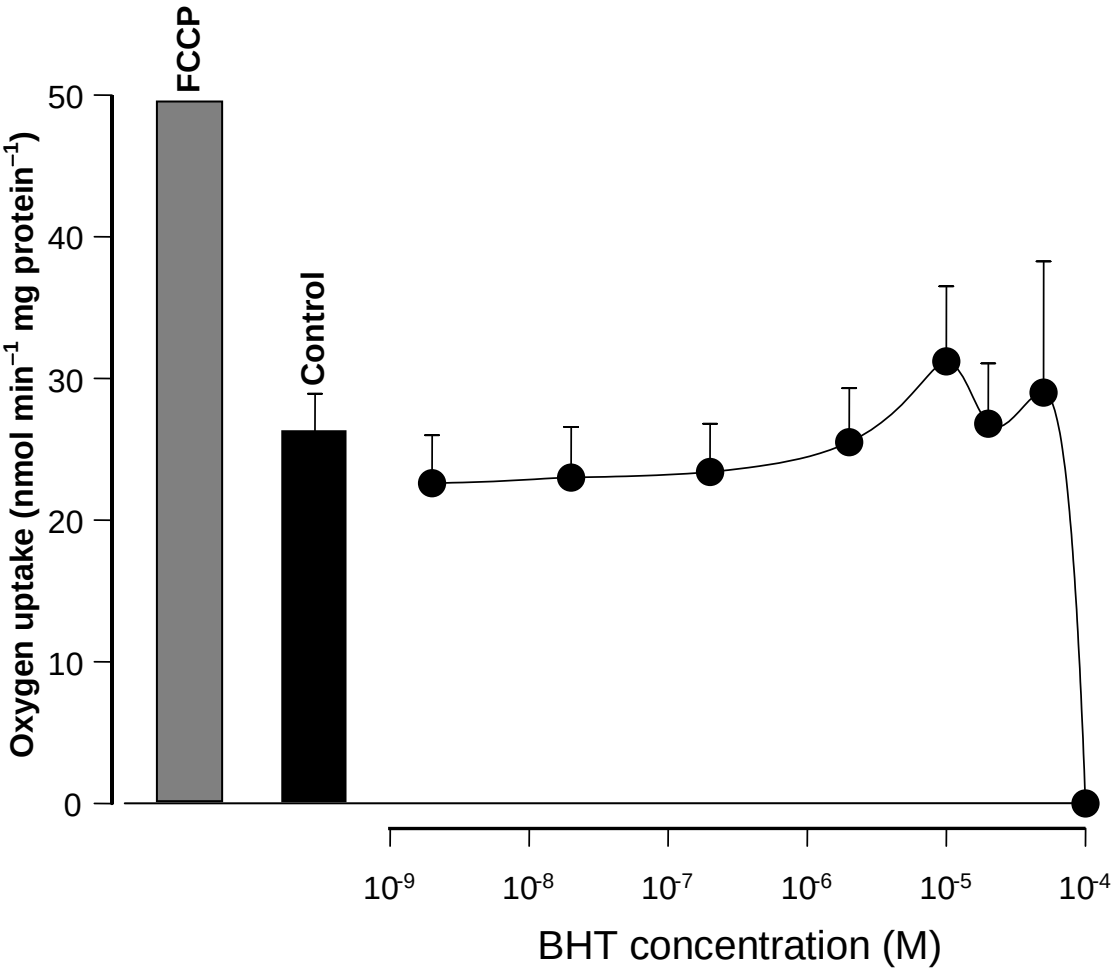
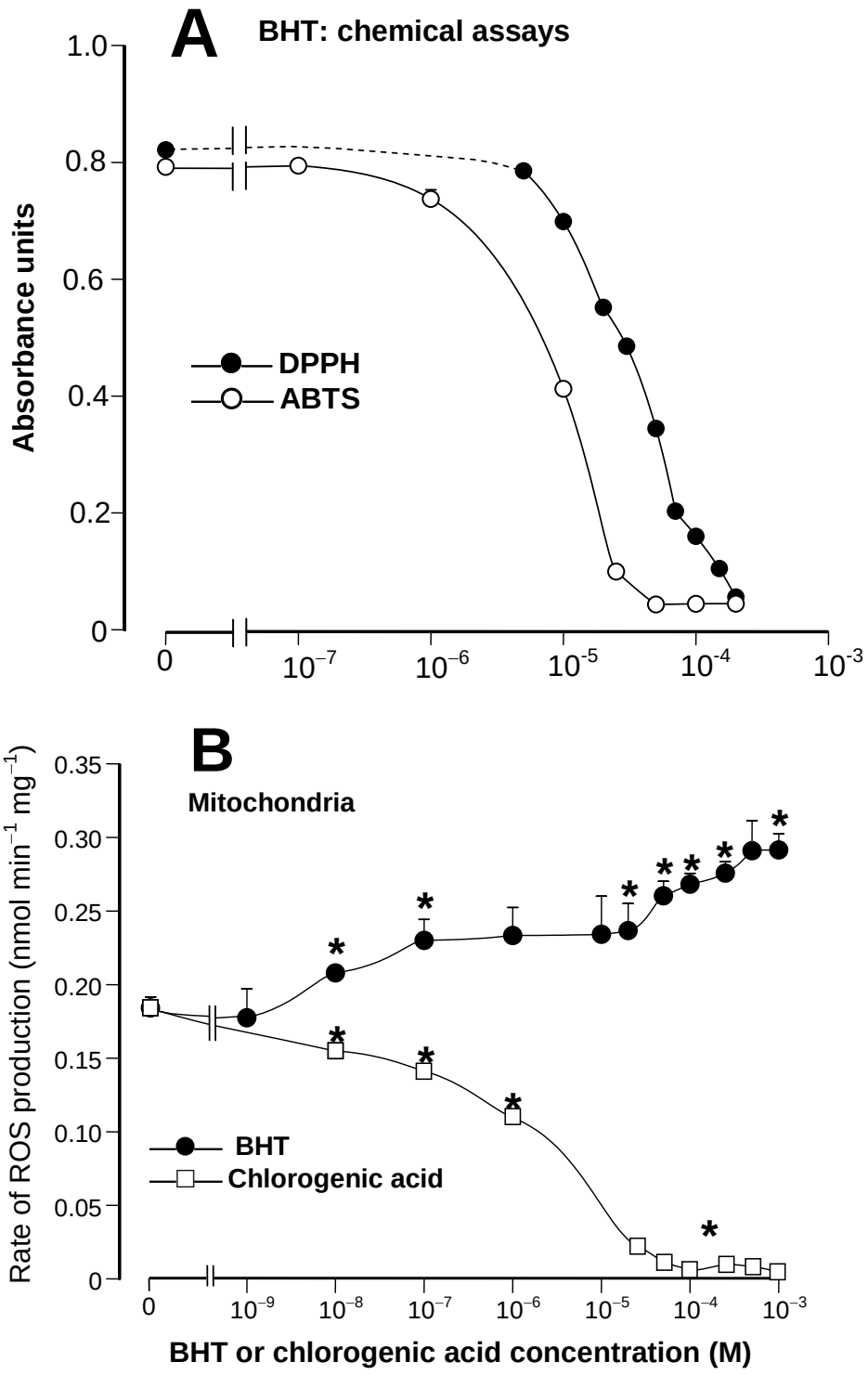

FCCP
Control
Oxygen uptake (nmol min−1 mg protein−1)
50
40
30
20
10
0
10-9
10-8
10-7
10-6
10-5
10-4
BHT concentration (M)

Figure 5


A
BHT: chemical assays
Absorbance units
1.0
0.8
0.6
0.4
0.2
0
DPPH
ABTS
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
B
Mitochondria
Rate of ROS production (nmol min⁻¹ mg⁻¹)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
BHT
Chlorogenic acid
0
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
BHT or chlorogenic acid concentration (M)

Artigo 2

Free radical scavenging activity of antioxidants in chemical systems and in isolated mitochondria

Lorena dos Santos Castro, Lívia Bracht, Anacharis Babeto de Sá-Nakanishi, Vanesa Gesser Correa, Rosane Marina Peralta and Adelar Bracht*
University of Maringá, Department of Biochemistry, 87020900 Maringá, Brazil

*Corresponding author:
Adelar Bracht
Department of Biochemistry
University of Maringá
87020900 Maringá, Brazil
Email: adebracht@uol.com.br; abracht@uem.br

Abstract

It is generally accepted that the generation of reactive oxygen species (ROS) in mitochondria is very important, perhaps even the most important one within most cells. The present work was planned to compare the free radical scavenging activity of several well known antioxidants in chemical systems with that in isolated rat liver mitochondria. The rate of mitochondrial ROS production was estimated by measuring the linear fluorescence increase due to 2'-7'-dichlorofluorescein (DCF) formation. The in vitro chemical antioxidant capacities were evaluated using both the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assays. Fourteen antioxidants were investigated, all capable of quenching free radicals in the ABTS and DPPH assays. Four of them clearly stimulated ROS production in rat liver mitochondria: butylated hydroxytoluene, butylated hydroxyanisole, ascorbic acid and gallic acid. Another group of efficient free radical scavengers in artificial systems were poorly active in the mitochondria, as for example propyl gallate and quercetin. The best free radical scavengers in the mitochondria, with IC_{50}'s smaller than 10 μM, were: resveratrol < chlorogenic acid < epicatechin < coumaric acid. The results reinforce the notion that demonstration of free radical scavenging activity for a given compound is no guarantee that it will act as an antioxidant in the mitochondria.

1. Introduction

It is generally accepted that the generation of reactive oxygen species (ROS) in mitochondria is very important, perhaps even the most important one within most cells (Murphy, 2009; see Figure 1). It is true that an accurate estimate of the contribution of the mitochondria for the total reactive oxygen species production within the cell is not yet possible. The same applies, however, for other organelles and compartments. Moreover, no other compartment presents such an extensive molecular network of chemical groups and reactions that greatly favor the formation of the oxygen superoxide ($O_2^{\cdot -}$; Murphy, 2009; Quinlan et al., 2012; Bleier and Dröse, 2013). It would, thus, be highly surprising if it turns out in the future that the contribution of the mitochondria is not one of the most important. Furthermore, the mitochondria possess their own superoxide dismutase (SOD), the enzyme that dismutases $O_2^{\cdot -}$ into H_2O_2, a strong argument in favour of a local production of $O_2^{\cdot -}$ (Figure 1). The mitochondrial ROS production can contribute significantly to damages to the organelles in several pathologies. On the other hand, it can also trigger important redox signalling cascades from the organelle to the rest of the cell (Droge, 2002); Balaban et al., 2005).

Several pathologies, such as arthritis and diabetes (Comar et al., 2013; Schubert et al., 2016; Biazon et al., 2016), for example, are accompanied by an overproduction of reactive oxygen species. The same occurs during aging in several tissues (Sá-Nakanishi et al., 2014). There is also an universal consensus that ingestion of molecular species able to scavenge free radicals (antioxidants) can, in part at least, prevent the deleterious effects of the reactive oxygen species (Sá-Nakanishi et al., 2014; Biazon et al., 2016). There are numerous molecular species able to scavenge free radicals including oxygen superoxide. In theory at least, good free radical scavengers should be active at the lowest possible concentration, allowing the ingestion of lowest possible doses. This is important because antioxidants are generally minor components of the daily diet and, consequently, ingested in relatively small quantities. The capacity of antioxidants in scavenging free radicals is generally evaluated using chemical assay systems. There is obviously no guarantee that a given substance active in scavenging free radicals in an artificial system will also be able to scavenge with the same efficiency the reactive oxygen species produced in the mitochondria, for example. In fact, in a previous work, we have found that butylated hydroxytoluene (BHT), a well known antioxidant (Llaurado, 1985; Horáková et al., 2000), far from acting as an

antioxidant in isolated rat liver mitochondria, did, on the contrary, stimulate the production of reactive oxygen species at very low concentrations (Castro et al., 2017). Taking into account this uncertainty, thus, the present work was planned to compare the free radical scavenging activity of several well known antioxidants in chemical systems with that in isolated mitochondria. The specific purposes were: (a) to find out if there are, besides BHT, other "antioxidants" able to act as "prooxidants" in mitochondria, i.e., as stimulators of ROS production; (b) to evaluate for those antioxidants that really act as such in mitochondria their potencies and compare them with the corresponding potencies in chemical systems.

2. Materials and methods

2.1. Materials

Bytulated hydroxytoluene, enzymes and coenzymes used in the enzymatic assays were purchased from Sigma-Aldrich Co (St. Louis, USA). All other chemicals were from the best available grade (98–99.8% purity).

2.2. Animals

Male Wistar rats weighing 200–280 g were used in all experiments. Animals were fed ad libitum with a standard laboratory diet (Nuvilab®, Colombo, Brazil) and maintained on a regulated light–dark cycle. All experiments were done in accordance with the worldwide accepted ethical guidelines for animal experimentation and were previously approved by the Ethics Committee of Animal Experimentatin of the University of Maringá (protocol n. 9120290915).

2.3. Mitochondria isolation

Fed rats were decapitated and their livers removed immediately and placed in ice-cold buffer containing 200 mM mannitol, 75 mM sucrose, 0.2 mM ethylene glycol tetraacetic acid (EGTA), 2 mM tris (hydroxymethyl)amino-methane (Tris–HCl), pH 7.4 and 50 mg% bovine serumalbumin. The tissue was minced, washed with the buffer and homogenized in the same medium by means of a van Potter homogenizer for lysing the cells. After homogenization, the mitochondria were isolated by differential centrifugation and suspended in the same medium, which was kept at 0–4 °C (Voss et al., 1961; Bracht et al., 2003).

Protein content of the mitochondrial suspensions was measured using the folin-phenol reagent and bovine serum albumin as standard (Lowry et al., 1951).

2.4. Mitochondrial reactive oxygen species (ROS) production

The rate of mitochondrial ROS production (real time ROS production), basically H_2O_2, was estimated by measuring the linear fluorescence increase (504 nm for excitation and 529 nm for emission) due to 2'-7'-dichlorofluorescein (DCF) formation from the reduced form of 2'-7'-dichlorofluorescein (DCFH) via oxidation by H_2O_2 in the presence of horseradish peroxidase (Zaccagnino et al., 2009; Comar et al., 2013; see Figure 1). Intact mitochondria (0.5 mg) were incubated in 2 mL of a medium containing 250 mM mannitol, 1.36 μM 2'-7'-dichlorofluorescein

acetate (DCFA-DA), 10 mM HEPES buffer (pH 7.2), 10 µM rotenone, 10 mM succinate as respiratory substrate and several antioxidants at various concentrations. The latter two were added as DMSO solutions. The acetate group of 2'-7'-dichlorofluorescein acetate is removed by endogenous esterases producing the reduced DCFH. Fluorescence due to 2'-7'-dichlorofluorescein (DCF) formation in consequence of ROS production was recorded during 10 min under agitation. The results were expressed as $nmol \cdot min^{-1} \cdot (mg \cdot protein)^{-1}$.

2.5. In vitro free radical quenching activity

The in vitro chemical antioxidant capacities were evaluated using both the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assays (Erel, 2004; Soares et al., 2009)

The cationic ABTS form was generated by incubating 3.7 mM ABTS with 1.3 mM potassium persulfate in the dark for 12 hours. After this time the incubation solution was diluted 60-fold with methanol. The assay system, 300 µL, was composed of 285 µL of the methanolic cationic ABTS solution (61 µM) plus 15 µL of DMSO solutions containing the antioxidant at appropriate concentrations. After one hour the absorbance decrease was measured at 734 nm.

The DPPH assay system (300 µL) was composed of 285 µL ethanolic 10.5 mM DPPH to which samples of 15 µL containing various concentrations of each antioxidants in DMSO were added. After one hour in the dark the absorbance at 515 nm was measured.

2.6. Data treatment

The error parameters presented in the graphs are standard errors of the mean. Computation of the IC_{50} values as done by numerical interpolation using Stineman's formula (Wagon, 1999). The software used was the *Scientist* program from MicroMath Scientific Software (Salt Lake City, UT, USA).

3. Results and discussion

All antioxidants utilized in the present work are listed in Table 1. The free radical scavenging activity was estimated using the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assays over a concentration range that allowed the construction of response-concentration curves of the type illustrated by Figure 2. All antioxidants produced response-concentration curves similar in form to those shown for quercetin in Figure 2. From each corresponding curve the concentration for half-maximal free-radical scavenging activity (IC_{50}) was computed by means of numerical interpolation (Stineman's formula; Wagon, 1999). In Table 1 the compounds were listed in order of their increasing IC_{50} values for the $ABTS^+$ scavenging activity, i.e., in order of decreasing ability. Gallic acid was the most effective $ABTS^+$ free radical scavenger, but ten compounds presented IC_{50} values in the range of up to 10 μM. Ascorbic acid was the least effective $ABTS^+$ free radical scavenger, more than ten times less effective than gallic acid.

IC_{50} values in the DPPH assay were in most cases higher with a few exceptions. The most notable exception was ascorbic acid, which was the most effective DPPH free radical scavenger. Vanillic acid was notable for its very poor activity in the DPPH assay, 45 times less effective than in the ABTS assay.

The direct measurement of the oxygen superoxide in mitochondria is hindered by its rapid dismutation to H_2O_2 by the superoxide dismutase present at very high molar concentrations 10 μM (Murphy, 2009). This leads to very low steady-state $O_2^{\cdot -}$ concentrations. For this reason, the production of $O_2^{\cdot -}$ is usually estimated by its dismutation product H_2O_2. If a free-radical scavenger is present, it can react with $O_2^{\cdot -}$, what results also in a smaller H_2O_2 output. In the present work, fluorescence due to DCF formation, which is proportional to the H_2O_2 concentration, was measured (see Figure 1). Response-concentration curves were obtained for all antioxidants listed in Table 1. Not all compounds presenting ABTS and DPPH free radical scavenging activities turned out to be net free-radical scavengers in isolated mitochondria. At least four of them considerably enhanced the net ROS production in mitochondria: BHT, BHA, gallic acid and ascorbic acid, as shown in Figure 3. As already reported, BHT increases ROS production starting at the concentration of 10^{-8} M (Castro et al., 2017). The concentration dependence was biphasic with a new increment starting at the concentration of 7×10^{-4} M and reaching 59% stimulation at the concentration of 10^{-3} M (Figure 3A). BHA also started to increase

the net ROS production at the concentration of 10^{-8} M (Figure 3B). The concentration dependence was not biphasic, however, and the final increment at 10^{-3} M was less pronounced than that caused by BHT (49%). Ascorbic acid (Figure 3C) increased only slightly the ROS production at the lowest concentrations, but from 10^{-5} until 10^{-3} M progressive increments occurred, reaching the final value of 44% stimulation. Gallic acid already showed maximal stimulation (62%) at the lowest concentration examined in the present work, namely 10^{-9} M (Figure 3D). This stimulation was maintained more or less constant over several orders of magnitude. Only at the concentration of 10^{-4} M the ROS production started to diminish. This diminution turned into inhibition as the concentrations were raised further and in the end an almost complete inhibition was found at the concentration of 5 mM (not shown, see Table 1 for the IC_{50} value). Figure 3 also shows the concentration dependences of four antioxidants that worked mainly as inhibitors of ROS production in mitochondria namely quercetin, resveratrol, propyl gallate and chlorogenic acid. The first two also presented small increments in ROS production at low concentrations. All other antioxidants investigated in the present work, however, presented only inhibition. Their IC_{50}'s are listed in Table 1. The four most effective antioxidants, with IC_{50}'s smaller than 10 μM were: resveratrol < chlorogenic acid < epicatechin < coumaric acid. Those with IC_{50}'s between 10 and 100 μM (10^{-4} M) were: rutin < caffeic acid < ferulic acid < propyl gallate < quercetin. Gallic acid and vanillic acid present IC_{50}'s in the millimolar range.

Simple examination of the data in Table 1 immediately suggests that a good correlation between the antioxidant activity in the chemical systems and the mitochondria cannot be expected. Some disparities are very pronounced. This is the case, for example, of ascorbic acid, which was the worst $ABTS^{+}$ free radical scavenger, the best DPPH free radical scavenger and a net stimulator of ROS production in the mitochondria. The expected poor correlation was indeed confirmed by the plots in Figure 4 and the corresponding linear regression analyses, whose results are given in the legend. It must be noted that correlation is poor even if extremes are withdrawn from the data set (not shown). These observations should not be surprising if one takes into account the conclusion of Alamed et al. (2009) that radical scavenging assays have limited value in predicting the ability of a compound to act as an antioxidant in complex foods.

The reasons why very effective free radical scavengers, such as gallic acid and several others, present either a poor performance in the mitochondria or are even

capable of stimulating ROS production is possibly linked to the complexity of the biological structures which allow a great number of interactions of various kinds. ROS production in the respiratory chain occurs at the FMN, FAD and CoQ levels, for example, whenever conditions are created to facilitate the transfer of one electron to the O_2 molecule (Murphy, 2009; Quinlan et al., 2012; Bleier and Dröse, 2013). These conditions may be created either by structural changes, binding phenomena or even by chanelling electrons into individual components of the respiratory chain. On the other hand, it is possible that those agents that stimulate ROS production in mitochondria actually exert a dual role: while acting as scavengers they also stimulate or create situations that lead to ROS production, the latter effect predominating over the first one over a certain concentration range.

It is already known that several of the compounds tested in the present work can exert a dual role, i.e., they can act as antioxidants under certain situations and in specific places or as prooxidants under distinct conditions. Yen et al. (2002), for example, found that gallic acid and ascorbic acid exert both antioxidant and prooxidant effects in human lymphocytes. They concluded that the prooxidant action of these compounds at lower concentrations may be due to their weak metal-chelating effects and their strong electron-donating effects (reducing ability), as well as to their stimulation of oxidative effects. In mitochondria the latter effect may be especially important for ascorbic acid, whose tendency in donating electrons to the respiratory chain is very pronounced, a phenomenon that can be extraordinarily accelerated by N,N,N,'N'-tetramethyl-*p*-phenylenediamine (TMPD) (Mustafa et al., 1968; Lofrumento et al., 1995). Another compound investigated in this work for which there are reports about its antioxidant and prooxidant activities is quercetin (Constantin and Bracht, 2008). However, in rat liver mitochondria and at least under the conditions of our assays, the compound revealed to be a ROS scavenger. To act as an antioxidant or as a prooxidant, thus, seems to depend not only on the specific conditions in a given biological system but also on the biological system with which the compound is interacting.

We found that both BHT and BHA increase ROS production in mitochondria. There is little doubt that these compounds are able to inhibit lipid peroxidation (Horáková et al., 2000; Bajpai et al., 2014), an action that they possible also exert in mitochondria. This action, however, is not incompatible with its stimulating action on ROS production derived from the superoxide anion production in the respiratory chain. Actually, lipid peroxidation initiates basically with the formation

of an alkyl radical (L·) (Alamed, 2008). Oxygen, in the ground state, behaves as a biradical due to its two unpaired electrons (·O–O·). The alkyl radical reacts rapidly with the oxygen biradical to form peroxyl radicals (LOO·), which may be transformed into hydroperoxides (Alamed, 2008). BHA and BHT may act as free radical quenchers in any of the various steps, thus inhibiting lipid peroxidation, a phenomenon that does not hinder their stimulating action on the reactive oxygen species ($O_2^{\cdot -}$) formation in the respiratory chain. It is difficult to infer about the mechanism by which BHT and BHA increase the reactive oxygen species formation in rat liver mitochondria. It is possible, however, that they do this by virtue of their interaction with components of the respiratory chain. Both compounds are uncouplers of oxidative phosphorylaton (Thompson and Moldéus, 1988), inhibitors of the respiratory chain (Festjens et al., 2006) and capable of interacting in a complex way with mitochondrial and lysosomal membranes (Sgaragli et al., 1971). Their stimulatory effects on ROS production occur at very low concentrations. This may be significant, because there are strong indications that at least the free BHT concentration within the cells is low to the point that the uncoupling effect in the perfused rat liver is barely detectable even at extracellular concentrations as high as 500 μM (Castro et al., 2017).

4. Conclusion

The results reinforce the notion that demonstration of free radical scavenging activity for a given compound is no guarantee that it will act as an antioxidant in the mitochondria, possibly the most important site of ROS production within the cellular environment. Some efficient free radical scavengers in artificial systems are poorly active in the mitochondria, as for example, propyl gallate and quercetin. Others may even increase ROS production, as for example ascorbic acid and gallic acid. It is important that the antioxidant activity is exerted at low concentrations, preferably in the micromolar range or below because the physiological concentrations are generally very low. Under this criterion, from the fourteen compounds investigated in the present work, only four can be expected to exert significant antioxidant activity in liver mitochondria under in vivo conditions, namely resveratrol, chlorogenic acid, epicatechin and coumaric acid.

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Table 1

Scavenging of the $ABTS^+$ and DPPH free radicals and inhibition of ROS production in isolated rat liver mitochondria by several antioxidants expressed in terms of their concentrations for half-maximal effect (IC_{50}).

Antioxidant	IC_{50} ABTS (μM)	IC_{50} DPPH (μM)	IC_{50} mitochondria (μM)
Gallic acid	2.72±0.15	8.49±0.13	2105.0±36.7*
Resveratrol	3.92±0.14	88.77±3.67	3.17±0.02
Quercetin	4.22±0.09	13.51±1.39	80.10±4.84
Propyl gallate	5.04±0.07	8.43±0.76	41.50±12.00
Epicatechin	5.74±0.02	9.11±0.32	7.77±0.64
Butylated hydroxyanisole (BHA)	6.77±0.11	19.16±0.80	Stimulation of ROS prodution
Ferulic acid	7.25±0.12	36.70±1.32	33.05±1.94
Coumaric acid	8.79±0.72	43.04±0.37	8.90±0.10
Butylated hydroxytoluene (BHT)	10.70±0.25	40.69±0.65	Stimulation of ROS prodution
Chlorogenic acid	15.14±0.76	7.75±1.02	4.63±0.52
Caffeic acid	16.33±1.06	47.12±0.07	18.34±1.2
Rutin	20.50±1.38	16.13±0.20	15.01±0.02
Vanillic acid	27.83±2.19	1517.3±157.0	2235.0±98.4
Ascorbic acid	33.18±0.24	5.14±1.14	Stimulation of ROS prodution

*Stimulation of mitochondrial ROS production at low concentrations.

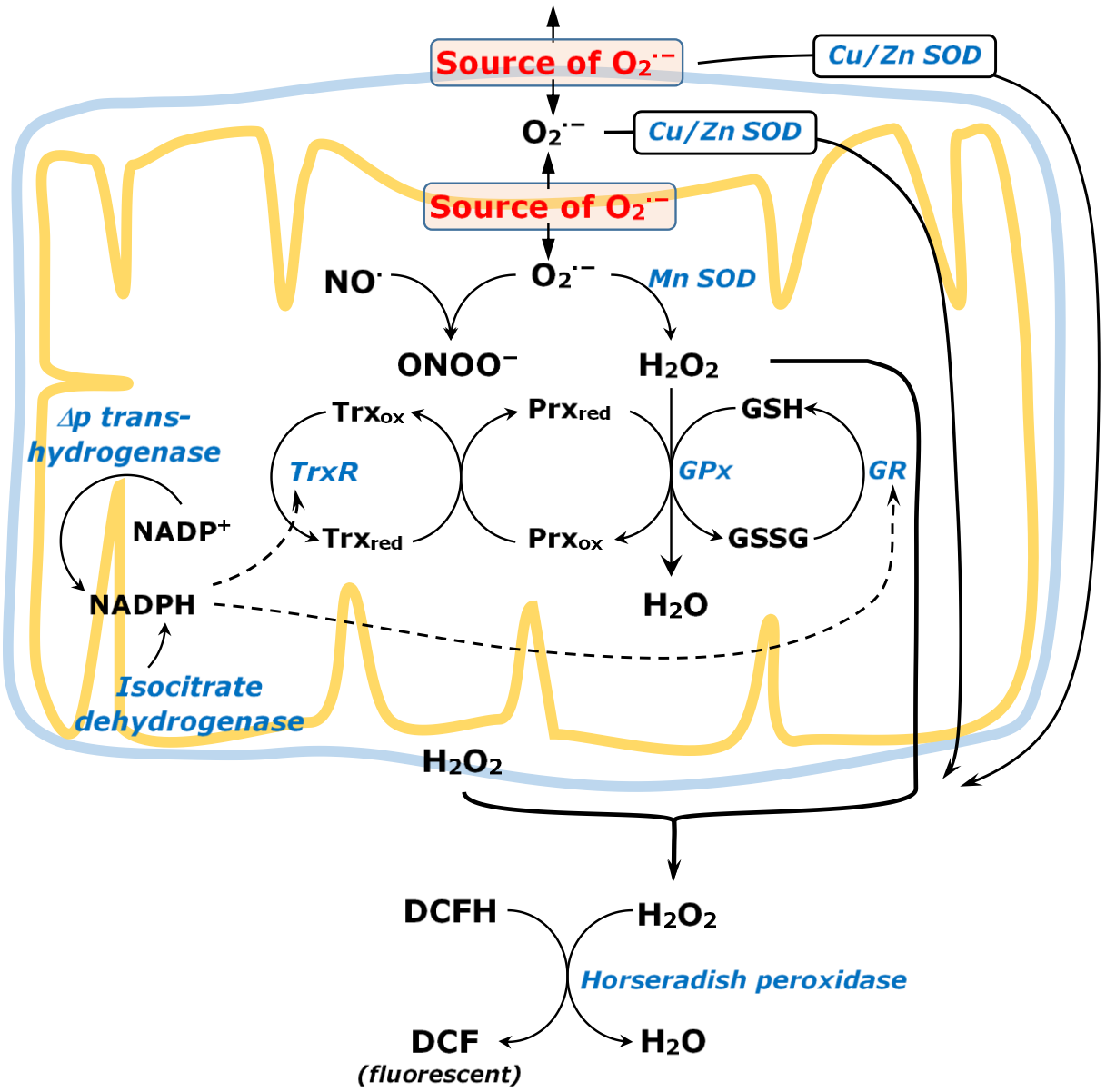

Figure 1. **Schematic representation of some mitochondrial events leading to $O_2^{\cdot-}$ and H_2O_2 production and the technique for measuring H_2O_2 production.** Symbols: GPx, glutathione peroxidase; Prx, peroxiredoxins; GR, glutathione reductase; Trx, thioredoxin; TrxR thioredoxin reductase-2; ox, oxidized; red, reduced; DCF, oxidized form of 2'-7'-dichlorofluorescein; DCFH, reduced form of 2'-7'-dichlorofluorescein.

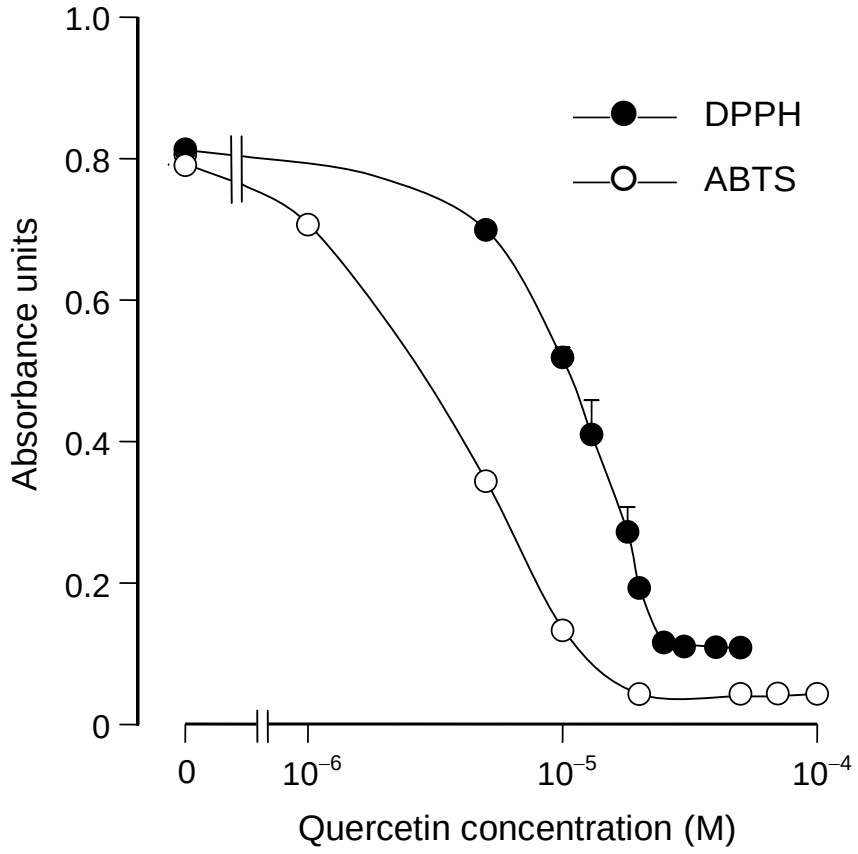

Figure 2. **Free radical scavenging activity of quercetin: concentration dependence.** The chemical free radical scavenging activities of quercetin were estimated as its capacity of scavenging the DPPH radical (1,1-diphenyl-2-picryl-hydrazyl) and of reacting with the ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) radical cation. The results were expressed as changes in absorbance at 515 nm for the DPPH assay and 734 nm for the ABTS assay.

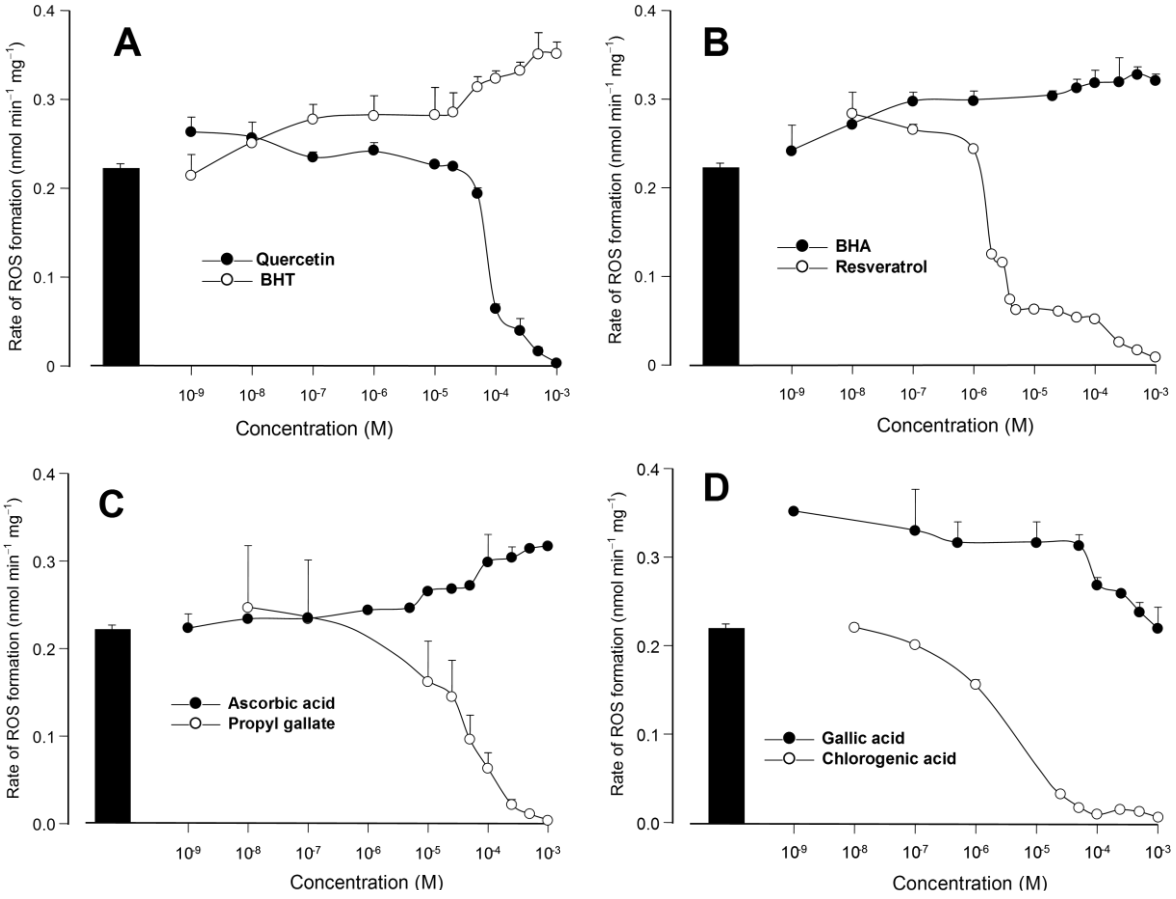

Figure 3. **Effects of several antioxidants on the mitochondrial generation of reactive oxygen species.** The rate of mitochondrial ROS production by freshly isolated mitochondria was estimated by measuring the linear fluorescence increase (504 nm for excitation and 529 nm for emission) due to oxidized 2′-7′-dichlorofluorescein (DCF) formation from the reduced form (DCFH) via oxidation by H_2O_2 in the presence of horseradish peroxidase. More details are given in the Materials and Methods section. Due to the logarithmic scale of the concentrations, the control value is given on the left by the dark bar. All values represent the mean of 3 determinations. Error parameters are standard errors of the mean (not visible when smaller than the symbol size).

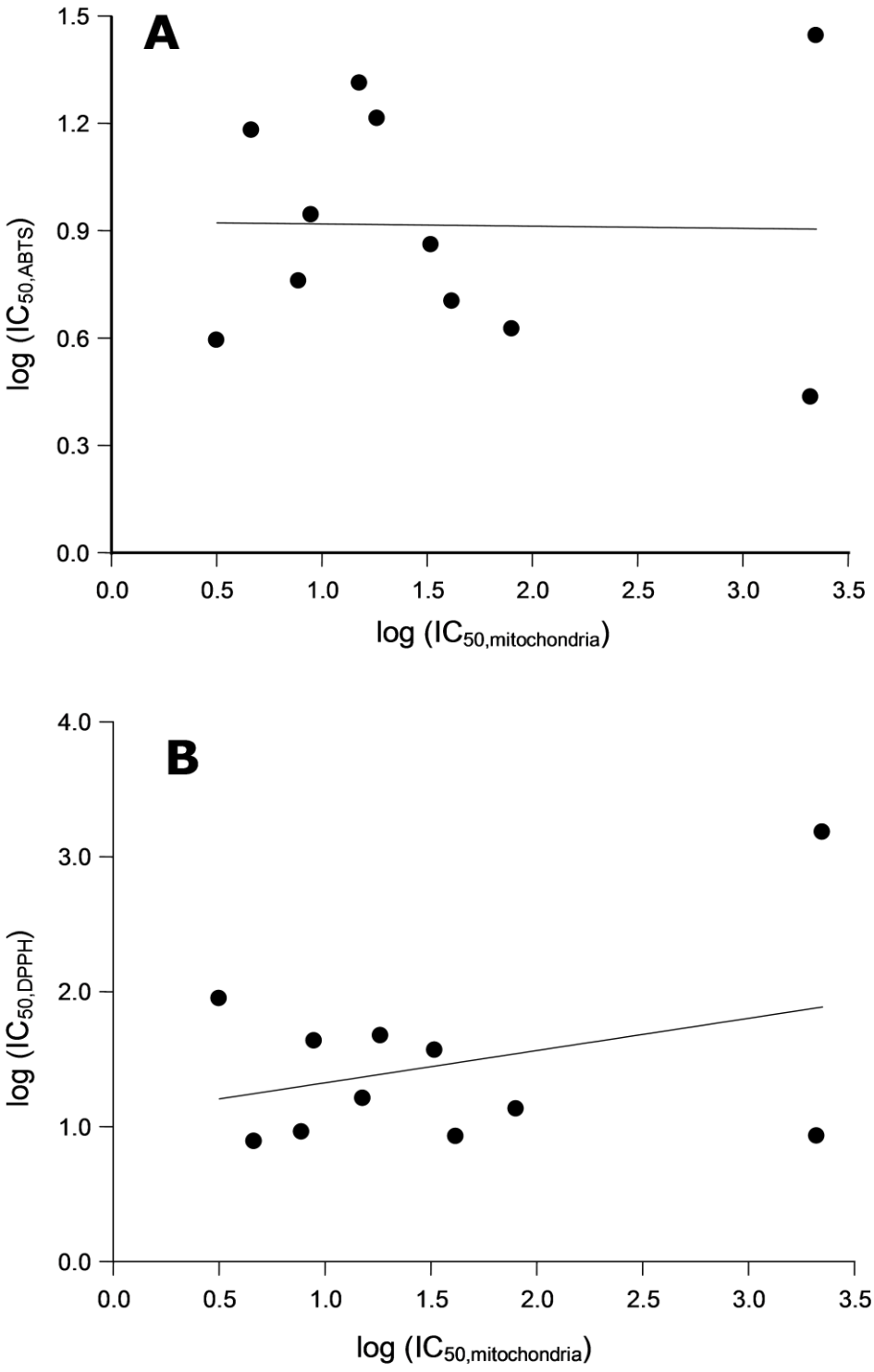

Figure 4. **Concentrations (µM) for half-maximal $ABTS^{+}$ (A) and DPPH (B) free radicals scavenging activities as a function of the concentrations (µM) for half-maximal diminution of ROS production in isolated mitochondria.** Experimental details are given in the Materials and Methods section. Double logarithmic scales were used because of the wide range of the various IC_{50} values (3 orders of magnitude). The lines represent the fitted linear correlation curve. (A): y = -0.00622x + 0.925 (r = 0.0182); (B): y = 0.239x + 1.0853 (r = 0.341).