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Short communication

Anethole dithiolethione prevents oxidative damage in glutathione-depleted astrocytes

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Abstract

Astrocytes protect neurons against reactive oxygen species such as hydrogen peroxide, a capacity which reportedly is abolished following loss of the antioxidant glutathione. Anethole dithiolethione, a sulfur-containing compound which is used in humans, is known to increase cellular glutathione levels and thought thereby to protect against oxidative damage. In the present study we found that anethole dithiolethione increased the glutathione content of cultured rat striatal astrocytes. This effect was abolished by coincubation with the glutathione synthesis inhibitor buthionine sulfoximine. Nevertheless, in the presence of buthionine sulfoximine, despite the lack of an increase in the lowered glutathione level, anethole dithiolethione fully protected the astrocytes against the enhanced toxicity of hydrogen peroxide. Thus, apparently other mechanisms than stimulation of glutathione synthesis are involved in the compound's protective action in astrocytes. Considering the occurrence of lowered glutathione levels in neurodegenerative syndromes, we conclude that further evaluation of the therapeutic potential of anethole dithiolethione is warranted. © 1997 Elsevier Science B.V.

Keywords: Anethole dithiolethione; Glutathione; Hydrogen peroxide; Oxidative stress; Astrocyte; Neurodegeneration

1. Introduction

Increased formation of reactive oxygen species and consequent oxidative stress is thought to be involved in the neuronal loss occurring in chronic neurodegenerative diseases and ischemia-reperfusion injury (Ames et al., 1993). Astrocytes, amongst other functions, are well known for their ability to protect neurons from oxidative stress, for instance in the form of exogenously applied hydrogen peroxide (H_2O_2, Ben-Yoseph et al., 1996; Langeveld et al., 1995). This phenomenon has been linked to the greater scavenging capacity of astrocytes as compared to neurons (Ben-Yoseph et al., 1996). Glutathione, the most abundant cellular free thiol, plays an important role in the inactivation of reactive oxygen species and is synthesized in large amounts in astrocytes (Langeveld et al., 1996). Moreover, loss of glutathione is accompanied by an enhanced susceptibility of astrocytes to oxidative damage and impairment of their neuroprotective action (Ben-Yoseph et al., 1996). Thus, considering the importance of glutathione in astrocyte-mediated neuroprotection, it is conceivable that compounds which enhance glutathione synthesis and/or restore declining glutathione levels in astrocytes will be of therapeutic benefit in neurodegenerative processes in which reactive oxygen species are implicated.

Anethole dithiolethione (5-(*p*-methoxyphenyl)-3*H*-1,2-dithiole-3-thione) belongs to a group of cyclic, sulfur-containing compounds collectively known as the 1,2-dithiole-3-thiones of which some are present in natural food stuff, in particular cruciferous vegetables (e.g., cabbage and Brussels sprouts, for review see Christen, 1995, although for criticism see Marks et al., 1991). Anethole dithiolethione has been in clinical use for decades as a choleretic and sialogogue without any major adverse reactions being noted. Interestingly, this highly lipophilic drug has been shown to increase cellular glutathione levels both in vivo and in vitro and to protect against (oxidative) damage incurred by loss of glutathione (Christen, 1995), at least outside the nervous system. Thus, taken this working profile together with its availability for use in humans, anethole dithiolethione may be ideally suited for develop-

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ment as a (new) drug in the treatment of oxidative stress-related brain disorders. However, to our knowledge, there is no information concerning the effect(s) of treatment with anethole dithiolethione on brain cell function. For the above-mentioned considerations, in order to start our evaluation of the potential of anethole dithiolethione to protect the brain from oxidative stress, we focussed the present investigation on the effect of anethole dithiolethione on glutathione levels and resistance against H_2O_2 toxicity in cultures of normal and glutathione-depleted astrocytes.

2. Materials and methods

2.1. Materials

Cell culture plastics were from Nunc (Roskilde, Denmark). Culture medium and supplements were from Gibco (Breda, Netherlands). Anethole dithiolethione was kindly provided by Solvay-Duphar (Weesp, Netherlands) through courtesy of Dr. E. Ronken. Stock solutions of anethole dithiolethione were prepared in dimethyl sulfoxide. Hydrogen peroxide (30%, w/v) was obtained from Mallinckrodt Baker (Deventer, Netherlands), whereas all other chemicals were from Sigma.

2.2. Cell culture

Cultures of astrocytes were prepared from the striatum of 1-day-old Wistar rat pups as described previously (Langeveld et al., 1995). Astrocytes were subcultured in 96-well or 12-well culture plates (coated with poly-L-lysine) at densities of 4×10^3 and 4×10^4 cells per well, respectively. As reported, under these conditions more than 95% of the cells in these cultures are identified as astrocytes (Langeveld et al., 1996). Biochemical assays were performed after the cells had been cultured for 7 or 8 days.

2.3. MTT assay

Drug effects on astrocyte viability were determined using a microculture tetrazolium assay, as described previously (Langeveld et al., 1995). Anethole dithiolethione (or vehicle) and/or L-buthionine-*S*,*R*-sulfoximine (BSO) were added to cultures grown in 96-well plates 24 h prior to the addition of H_2O_2. Following an additional period of 24 h, the assay was started by a 2 h incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; 50 μg/well). Subsequently, after solubilization by vibration on a plate shaker, the amount of formazan formed was quantified by measuring the absorbance at 540 nm using a microculture plate reader. The viability in treated cultures was expressed as a percentage of the mean absorbance in control (i.e., vehicle-treated) cultures, after subtraction of mean background absorbance (determined in wells without cells).

2.4. Glutathione assay

Astrocytes cultured in 12-well plates were used for spectrophotometric determination of total glutathione (i.e., reduced glutathione + 2 × glutathione disulfide; GSx) content. Following a 24 h incubation with anethole dithiolethione (or vehicle) and/or BSO, the assay, including determination of protein content, was performed as described previously (Drukarch et al., 1996).

3. Results

Prior to evaluating its protective potential in our culture system, we assessed the toxicity of anethole dithiolethione using the MTT assay. Our data showed a significant reduction in astrocyte viability following a 48 h exposure to anethole dithiolethione in concentrations of 100 μM or more (data not shown). Moreover, in concordance with observations by others (Sen et al., 1996), under these conditions formation of crystals was detected upon microscopic examination. Since neither toxicity nor crystallization occurred in concentrations up to 30 μM, we decided to use the compound in final concentrations not higher than this. A 24 h incubation with anethole dithiolethione, in concentrations of 10 and 30 μM, induced a statistically significant and concentration-dependent increase in GSx content (Fig. 1). Conversely, 24 h treatment with the glutathione synthesis inhibitor BSO (100 μM) reduced GSx levels to approximately 10% of that in untreated cultures (Fig. 1). Moreover, in the presence of BSO, anethole dithiolethione failed to increase GSx content above

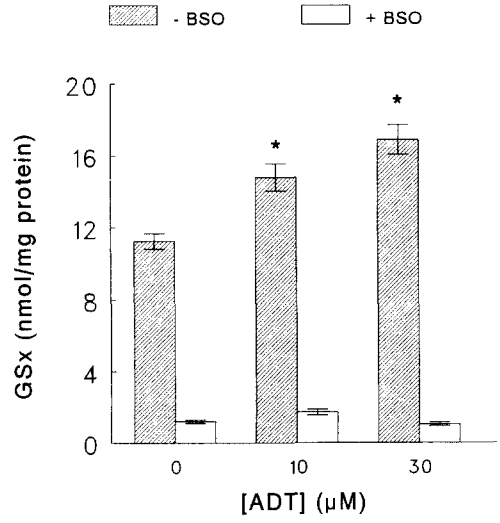

Fig. 1. Effect of incubation with anethole dithiolethione (ADT) on total glutathione (GSx) content in cultured rat striatal astrocytes in the absence (hatched bars) or presence (white bars) of buthionine sulfoximine (BSO, 100 μM). GSx content was determined 24 h after addition of anethole dithiolethione and/or BSO. Values represent mean ± S.E.M. of four independent experiments performed in triplicate. * Different from control (without anethole dithiolethione), $P < 0.001$ (Student's *t*-test).

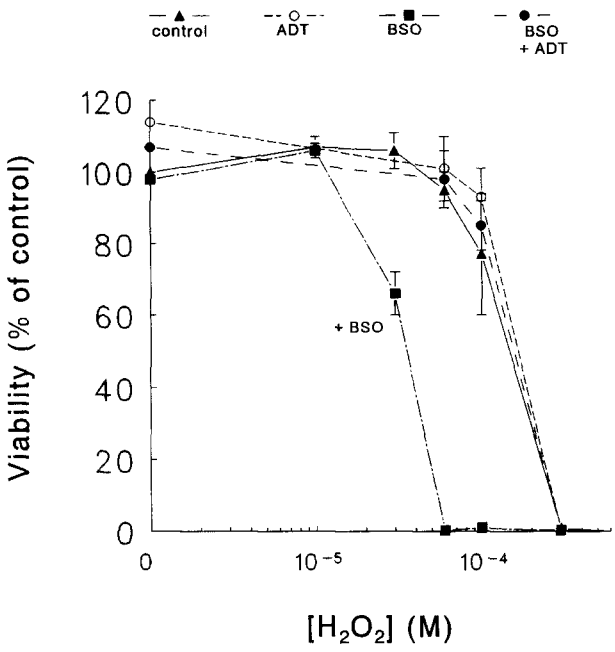

Fig. 2. Effect of hydrogen peroxide (H_2O_2) on the viability of cultured rat striatal astrocytes in the presence of buthionine sulfoximine (BSO) and/or anethole dithiolethione (ADT). BSO (100 μM) and anethole dithiolethione (10 μM) were added 48 h and H_2O_2 24 h prior to estimation of viability. Values are the mean ± S.E.M. of three independent experiments in which in each experiment six wells/group were used. Data are expressed as percentage of the absorbance in untreated (i.e., astrocytes not incubated with either BSO, anethole dithiolethione and/or H_2O_2), control cultures.

that measured in astrocytes treated with BSO alone (Fig. 1).

Loss of glutathione in astrocytes incubated with BSO (100 μM) was found to coincide with a substantially enhanced toxicity of H_2O_2 (10–300 μM) as compared to non-glutathione-depleted control cells (Fig. 2). Although pretreatment with anethole dithiolethione did not improve the resistance to H_2O_2 of non-glutathione-depleted control cells (Fig. 2), the compound, added simultaneously with BSO, prevented the increased toxicity observed in glutathione-depleted astrocytes. Our data demonstrated that full protection against the enhanced toxicity of H_2O_2 in glutathione-depleted astrocytes was provided by 10 μM anethole dithiolethione (Fig. 2).

4. Discussion

Our data show that in vitro treatment with anethole dithiolethione, in concentrations (estimated by blood plasma sampling) in which the compound is able to prevent (per)oxidative damage in vivo in rodents (Dansette et al., 1990), induced an increase in the glutathione content of cultured astrocytes. This effect, reaching approximately 145% of control values at 30 μM ADT, was comparable to that reported by others in a T-lymphocyte cell line, albeit at a somewhat higher concentration (100 μM; Sen et al., 1996). As mentioned above, under our experimental conditions poor solubility and toxicity precluded the use of anethole dithiolethione in concentrations of 100 μM or more. Since our data furthermore demonstrate that treatment with anethole dithiolethione failed to prevent the loss of glutathione incurred by addition of the γ-glutamylcysteine synthetase inhibitor BSO (Griffith, 1982), it seems most likely that the compound mediated the increase in glutathione content by stimulation of synthesis and not by inhibition of its breakdown and/or efflux from the cells. Indeed, elevation of γ-glutamylcysteine synthetase activity, the rate-limiting enzyme in glutathione synthesis (Griffith, 1982), has been noted ex vivo in liver homogenates subsequent to administration of anethole dithiolethione (Davies et al., 1987). Nevertheless, in contrast to what has been suggested previously (Christen, 1995; Sen et al., 1996), our results argue against a direct link between the anethole dithiolethione-induced increase in glutathione content and protection against oxidative insults, at least in astrocytes. Thus, despite the increase in glutathione levels, no change in susceptibility to H_2O_2 toxicity was detected in cultures treated solely with anethole dithiolethione. In agreement with data obtained by others (Ben-Yoseph et al., 1996), glutathione depletion with BSO was accompanied by an enhanced reduction of astrocyte viability in the presence of H_2O_2. Under these conditions, however, apparently without increasing glutathione content, anethole dithiolethione was able to restore astrocyte resistance to H_2O_2 back to the level of non-glutathione-depleted cells. The lack of a protective effect in non-BSO-treated astrocytes moreover indicates the absence of a direct (extracellular) scavenging of H_2O_2 or, considering the possibility of a Fenton reaction in our transition metal-containing culture medium, the more reactive hydroxyl radical by anethole dithiolethione.

Anethole dithiolethione, like other dithiolethiones, elevates the activity of enzymes such as glutathione disulfide (GSSG) reductase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase (Christen, 1995; Davies et al., 1987). These dehydrogenases form part of the pentose phosphate pathway providing NADPH which is vital for GSSG reductase function. Interestingly, the pentose phosphate pathway is stimulated by the addition of H_2O_2 to astrocytes, which effect is counteracted by BSO-induced depletion of glutathione leading to enhanced toxicity (Ben-Yoseph et al., 1996). Therefore, it seems feasible that anethole dithiolethione, through an increase in the activity of the pentose phosphate pathway and/or GSSG reductase and the resulting enhanced efficacy of glutathione redox cycling (Ben-Yoseph et al., 1996), is able to overcome the consequence(s) of a reduction in glutathione levels. Furthermore, anethole dithiolethione, in concentrations comparable to those used by us, has been shown to be a potent inhibitor of lipid peroxidation in preparations of rodent liver, thereby possibly adding to its protective action (Mansuy et al., 1986).

Signs of oxidative damage have been detected in most if not all neurodegenerative syndromes and loss of glutathione has been noted in particular in Parkinson's disease and ischemic brain damage (Ames et al., 1993). Thus, although characterization of the mechanism(s) of action is certainly warranted and despite the fact that data obtained by an in vitro approach may not necessarily be predictive for the in vivo situation, considering that anethole dithiolethione has been used in humans for a considerable number of years without major adverse effects our results provide a strong incentive for further evaluation of the therapeutic potential of the compound in named conditions.

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