Pro- and Antioxidative Properties of Cortical Tissue Preparations from Human Brain Exhibiting NMDA-Receptor Characteristics

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The effects of cortical tissue preparations (CTP) from human brain on the production of reactive oxygen species (ROS) has been investigated with several biochemical model reactions. As indicators for ROS, fragmentation of the methionine derivatives, α-keto-γ-methylthiobutyric acid (KMB) or 1-amino-cyclopropane-1-carboxylic acid (ACC), yielding ethene have been used. With these systems we have shown that production of OH-radical-type oxidants by the xanthine oxidase (XOD)-system is strongly stimulated by CTP. This activity is due to intrinsic iron ions since ethene formation from KMB is stimulated by EDTA, inhibited by desferrioxamine (Desferal^R) and also visible with heat-denatured CTP. CTP by themselves have no XOD activity.

3-Hydroxykynurenine (3HK) is another possible substrate for XOD but produces H_2O_2 without XOD-catalysis, whereas allopurinol is not inhibiting. CTP contain measurable NAD(P)H oxidoreductase activity, producing OH- radical- type oxidants at the expense of NADPH and (to a lesser extent) NADH as electron donors , shown as redox-cycling of 2-methyl-5-hydroxy-1.4-naphthoquinone, plumbagin. Ethene formation from KMB is also driven by both morpholinosydnonimine (SIN) or ONOOH. The reaction driven by SIN is stimulated by CTP and inhibited by catalase, SOD and hemoglobin. Since ethene release from KMB driven by ONOOH is inhibited by CTP the mechanisms driving KMB fragmentation are different for SIN and ONOOH.

Furthermore CTP contain approx. 4 U catalase activity per mg protein and very weak peroxidase (POD) activity shown as ACC fragmentation yielding ethene in the presence of both H_2O_2 and KBr or NaCl. Since ACC binds to CTP and both compounds, ACC and KMB are natural products, present in food (ACC) or synthesized from methionine *in vivo* (KMB), these compounds may represent protecting agents in systems where reactive oxygen species are formed. One might even speculate that the production of ethene at these membrane receptor sites may have biological functions, since ethene is known to possess anaesthetic activities.

Introduction

Several neurological disorders have been causally connected with activated oxygen species produced by several mechanismns in different cells and various tissues. The CNS has been addressed as "particulary vulnerable to free radical damage". In a recent review Knight (1997) mentions several plausible reasons favoring oxidative damage of the CNS, namely, strong oxidative metabolic activity, low concentrations of antioxidants, high concentrations of polyunsaturated fatty acids and endogenous generation of oxygen radicals. There are also numerous reports on the involvement of ROS in neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease, amyotrophic lateral sclerosis, multiple sclerosis, Downs Syndrome, as well as mitochondrial DNA disorders and ischemia-reperfusion damage (Gerlach *et al.,* 1996).

Principally, one can differentiate between transition metal-catalyzed production of strong oxidants (iron or copper catalysis) producing Fenton-type

Abbreviations: NMDA, N-methyl D-aspartate; ACC, 1-amino-cyclopropane-1-carboxylic acid; KMB, α-keto-γ-methylthiobutyric acid; CNS, central nervous system; ROS, reactive oxygen species; SOD, superoxide dismutase; XOD, xanthine oxidase; SIN-1, 3-morpholinosydnonimine; MPO, myeloperoxidase; Hb, hemoglobin; POD, peroxidase; MAO, monoamine oxidase; CTP, cortical tissue preparations; 3 HK, 3-hydroxykynurenine.

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oxidants, hypohalous acids, such as HOCl or HOBr , and peroxynitrite, originating from the reaction between superoxide and NO (Halliwell, 1992; Hippeli *et al.*, 1997, van Dyke, 1997). In addition, autooxidizable Amadori-products derived from protein glycation, i.e. derivatization by reaction with the aldehyde group of glucose, have to be envisaged as an important source of ROS during neurodegeneration (Muench *et al.,* 1996).

Potential producing sites of ROS in CNS may be represented by activated cells such as microglia (Hu *et al.,* 1996), dysfunctioning organelles such as mitochondria (Fukushima *et al.,* 1995, Dugan *et al.,* 1995), and enzymatic reactions, such as MAO (Olanow, 1993, Ebadi *et al.,* 1995), XOD (Aizenman, 1995; Okuda *et al.,* 1996; Atlante *et al.,* 1997; Fachinetti *et al.,* 1992) or NOS (Dawson and Dawson, 1996, Paakari and Lindsberg, 1995). There are also recent reports on ROS production by activated NMDA-receptors (Fagni *et al.,* 1994; Gunasekar *et al.,* 1995). NMDA-receptors exhibit different binding domains where besides NMDA, glutamate and amino acids such glycine and the non proteinogenic cyclic amino acid, ACC, have been described. ACC and KMB are derivatives of the sulfoaminoacid, methionine and both, ACC and KMB are precursors of the plant hormone ethene, where ACC is predominatedly fragmented by hypohalous acids and chloroamines, while KMB is mainly fragmented by Fenton-type oxidants (von Kruedener *et al.,* 1995), or peroxynitrite (Hippeli *et al.,* 1997).

In the present communication we report on intrinsic prooxidative and antioxidative capacities of human cortical tissue preparations, further characterized as to contain a glycine site associated with N-methyl-D-aspartic acid receptors, taking advantage of the above-mentioned differential oxidative fragmentation of either ACC or KMB as sensitive indicators for the production of ROS, or its inhibition.

The following ROS or ROS- generating systems have been used in order to investigate on either stimulating or inhibiting effects of CTP in these biochemical models.

1. XOD: Xanthine oxidase is supposed to play an important role during reperfusion damage (Halliwell and Gutteridge, 1989; Atlante *et al.,* 1997; Fachinetti *et al.,* 1992). As substrates, aldehydes, pteridines, hypoxanthine and xanthine have been described where allopurinol functions as a suicide substrate. During ischemic periods native xanthine dehydrogenase reducing NAD^+ at the expense of the above substrates is converted to an oxidase, producing superoxide, hydrogen peroxide and due to intrinsic iron, also the OH-radical. As a test system for OH-production ethene release from KMB was chosen.

2. NAD(P)H oxidoreductases: NAD(P)H oxidoreductases, frequently also addressed as diaphorases, transfer electrons between NAD(P)H and various electron acceptors. Some of these enzymes are part of NAD(P)H oxidases producing ROS. Non autooxidizable diaphorases may be rendered oxidizable by coupling to an oxidizable redox cofactor such as various naphtho- and anthraquinones. Such systems produce superoxide and hydrogenperoxide. Production of OH-radicals in these systems may occure independent of transition metals since, semiquinones as one-electron transition product may act as electron donors for H_2O_2.

3. Peroxynitrite (ONOOH): Several types of cells, such as endothelial cells, neutrophils and microglia, produce both superoxide and NO which interact extremely rapid ($k=7*10^9$) under formation of ONOOH. ONOOH in turn has oxidative properties similar to Fenton-type oxidants, which can be differentiated by several radical scavengers and chelators such as EDTA (Hippeli *et al.,* 1997). In addition to this it has been shown to form nitro derivatives with several aromatic ring systems, such as tyrosine. Involvement of peroxynitrite in neurotoxicity in context with NMDA-receptors has been reported by several groups (Beckman *et al.,* 1994; Fagni *et al.,* 1994; Paakkari and Lindsberg, 1995; Ohkuma *et al.,* 1995; Gunasekar *et al.,* 1995; Dawson and Dawson, 1996). Experimentally ONOOH is produced by SIN-1, a compound which in aqueous solutions simultaneously releases NO as well as superoxide (Bohn and Schönafinger 1989, Feelisch *et al.,* 1989).

It has been shown, that peroxynitrite similar to Fenton-systems is able to release ethene from KMB (Pryor and Squadrito, 1995). In a similar way KMB-fragmentation is driven by both SIN-1 or chemically synthesized ONOOH (Hippeli and Elstner 1997).

4. Myeloperoxidase: MPO in the presence of chloride and H_2O_2 produces HOCl, which rapidly

reacts with ACC, yieling ethene (v. Kruedener *et al.*, 1995; Albrecht-Goepfert *et al.*, 1998). Commercially available horseradish peroxidase (HrPOD) fragments ACC in the presence of H_20_2 and KBr but not in the presence of NaCl (Heiser *et al.*, 1999, in press). We repeated this experiment with HrPOD in comparison to CTP.

Materials and Methods

Membrane preparation: The membranes used for this study were prepared from human cortex (Gsell *et al.*, 1993) essentially as described by Kessler *et al.*, 1989. Briefly, cortex samples were homogenized in 320 mM Sucrose and 1 mM EGTA/ Tris- (ethylene glycol-bis-(β-aminoethyl ether) / trishydroxymethyl aminomethane) buffer (pH 7,0); (1:10/w:v). The P2 fractions were prepared by differential centrifugation (1000 g for 10 min.; 35000×*g* for 20 min), resuspended in 20 ml of lysis solution (1 mM EGTA/Tris, pH 8,0), sparing the brown, mitochondria-enriched core of the pellet and allowed to lyse for 15 min. on ice. The CTP were spun down at 40000×*g* for 30 min. The pellet was resuspended in lysis buffer (again sparing the brown core) and recentrifuged. The resulting pellet was suspended in Tris/acetate buffer (100 mM Tris/acetate and 50 μM EGTA; pH 7.4) to give a concentration of 1 mg protein/ml. After Triton-X-100 was added (to a final concentration of 0.5%), the CTP were left at 0 °C for 30 min, centrifuged at least three times to remove the detergent, and resuspended to a final concentration of 1–2 mg of protein/ml in Tris/ acetate buffer. Membranes were frozen at −70 °C.

The membrane preparations were characterized by competitive binding assays utilizing H^3-glycine and unlabeled ACC according to the method described by Marvizon *et al.*, 1989. Protein concentrations were determined according to Bradford (1976).

Pro- or antioxidative activities were determined as described by von Kruedener *et al.* (1995), Albrecht-Goepfert *et al.* (1998) and Hippeli *et al.* (1997).

Superoxide, hydrogen peroxide and OH-radical-type oxidant and hypohalides were produced by the XOD-, diaphorase- or MPO- systems. Peroxynitrite was either produced by aqueous solutions of SIN-1 (Feelisch *et al.*, 1989), or by synthesis from KNO_2 and hydrogen peroxide according to Beckman *et al.* (1994). Ethene formation from ACC or KMB was followed gaschromatographically by the "head-space-technique" (v. Kruedener *et al.*, 1995). Further experimental details are outlined in context with the individual tables and figures. The presented results are means of three individual experiments undertaken on two different days (n=6). Standard deviations are given as σ (n-1).

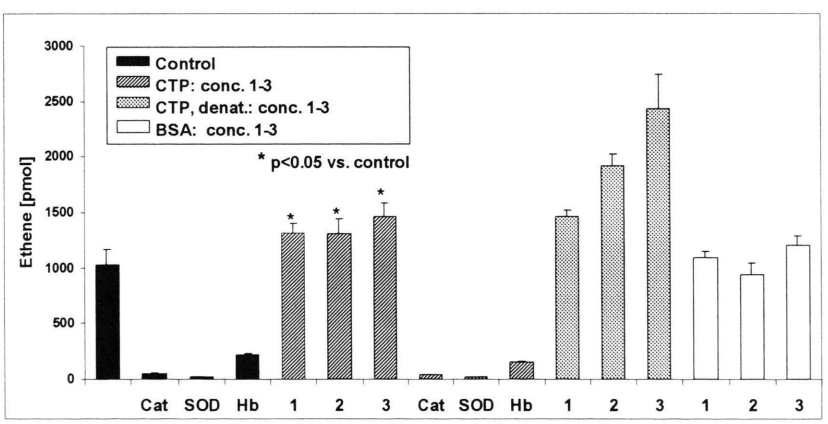

Fig. 1. Effects of CTP on xanthine oxidase-driven ethene release from KMB.
The test system contained in 2 ml: 0.1 M Tris-buffer pH 7.4; 1.5 mM KMB, 0.5 mM xanthine, 0.8 units XOD, three different concentrations of CTP or BSA (where the concentrations 1, 2 and 3 correspond to 0.2, 0.4 or 0.6 mg/ml protein, respectively), catalase (Cat, 100 U), SOD (100 U) or hemoglobin (Hb, 50 μM) as indicated; CTP was denatured by boiling for 5 min; The reaction mixture was incubated for 30 min in the dark at 37 °C; After the incubation 1 ml of gas was withdrawn from the head space of the test vessels through a gas-tight rubber tab. Ethene-concentrations were analysed gas chromatographically, as described by v. Kruedener *et al.*, 1995.

Results and Discussion

Production of ROS by XOD

As shown in Fig. 1 ethene release by the XOD-system is stimulated by our cortical synaptosomal preparation. This, however, is not due to enzymatic activity, since denaturation of CTP at 100 °C yields much stronger stimulation. In the absence of either the substrate xanthine or XOD no ethene formation can be measured (data not shown). Both in the absence or in the presence of CTP, catalase and SOD (100 U each) inhibit KMB-fragmentation by more than 95% while Hb (50 μM) inhibits by approx. 80%. EDTA stimulates ethene release, both in the control system and in the XOD system in the presence of CTP whereas desferal is a strong inhibitor. Since EDTA augments Fe^{2}-Fe^{3} redox transitions and desferrioxamine (Desferal^{R}) is a strong iron chelator inhibiting this transition we have to assume that CTP contains bound iron, not identical to hemoglobin (Fig. 2), since Hb inhibits this reaction (see Fig. 1).

The neurotoxin 3-hydroxykynurenine (3HK) in concentrations of higher than 500 nM, in agreement with Okuda *et al.* (1996), can act as a substrate for XOD. Under our experimental conditions, however, 3HK to some extent is also active in the absence of XOD. The reaction mechanism, however, is different to the one of other substrates, since SOD, EDTA and allopurinol are not inhibitory, whereas catalase and desferal are inhibitors both in the presence and in the absence of XOD. Since no intrinsic XOD activities could be measured in our membranes, XOD as an oxygen radical source in CTP is unclear in this respect (Figs 3, 4).

NAD(P)H oxidoreductases

As shown in Fig. 5 commercially available diaphorase produces ethene from KMB in a reaction which is dependend on both NADPH and plumbagin as autooxidizable naphthoquinone redoxcycler. CTP (0.36 mg) exhibits approximately 40% of the activity measured with 5 units of the commercial enzyme. A similar, but much weaker response was obtained with NADH as electron donor, indicating that this activity was not due to mitochondrial enzymes. The measureable activity of our preparation seems to be due to a proteinaceous catalyst, since boiling completely abolishes the activity. Our results indicate, that CTP contain NADPH-diaphorase-active proteins, which are not autooxidizeable, however (no activity without plumbagin). Production of ROS by this protein(s) is dependent on the presence of appropriate redoxcyclers. Since a vast amount of natural and synthetic compounds have been shown to exhibit such activities the function in the brain seems to be dependent on the penetration of the brain blood barrier of such compounds.

Peroxynitrite ($ONOOH$, $ONOO^{-}$) as destructive ROS

As shown in Fig. 6, SIN-1 driven ethene release from KMB is inhibited by SOD, catalase and he-

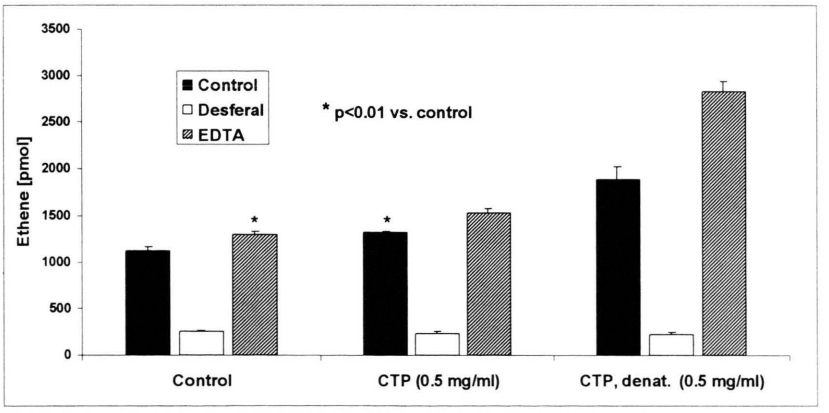

Fig. 2. Effects of EDTA or desferal on ethene release from KMB during the XOD reaction. Reaction conditions were identical to those described in Fig. 1, except that 0.5 mM EDTA or 76 μM desferal were added as indicated. 0.5 mg /ml CTP or denatured CTP were used.

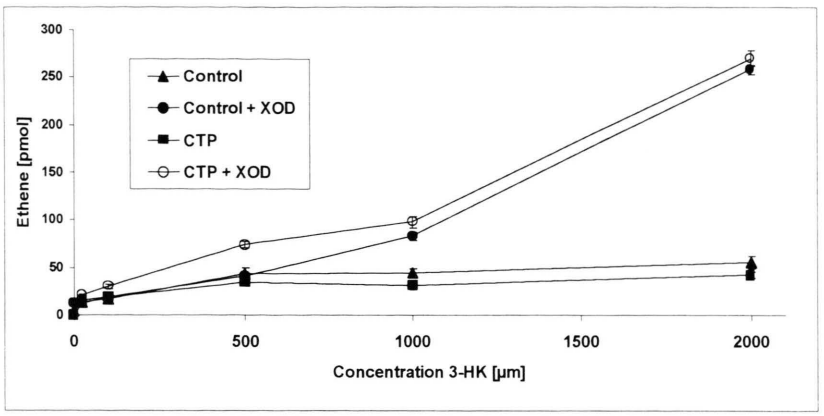

Fig. 3. Ethene release from KMB by 3HK in the absence or presence of XOD.
The reaction conditions were identical with those in Fig. 1, except that different amounts of 3HK were present as substrate instead of xanthine.

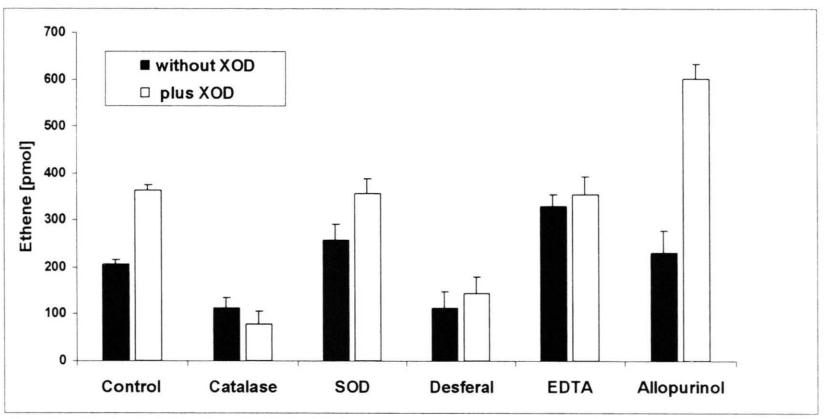

Fig. 4. Effects of catalase, SOD, desferal, EDTA or allopurinol on ethene release from KMB driven by 3HK in the absence or presence of XOD.
The reaction conditions were identical with those in Fig. 1, except that 1 mM 3HK was used as substrate instead of xanthine and 100 U of catalase or SOD, 0.5 mM EDTA, 76 µM Desferal or 300 µM allopurinol were added as indicated.

moglobin and desferal but stimulated by our membrane preparation. Inhibition by the above substances in the presence of CTP is identical to the control. Since EDTA shows no stimulating effect, inhibition by desferal is not due to iron-chelating, but more likely to unspecific reaction with SIN-1 or its degradation products. In contrast to the SIN-1 reaction ethene release from KMB by ONOOH, however, is inhibited by more than 50% by EDTA, desferal and CTP (data not shown). Therefore, the stimulatory effect of CTP in the SIN system represents another effect than just enhancing the activity of ONOOH. Since SIN-1 in contrast to synthetic ONOOH is more likely to represent physiological conditions an enhancement of the production of a strong oxidant from superoxide and NO by this membrane preparation and thus an enhancement of NO and superoxide toxicity has to be envisaged.

Myeloperoxidase activity

HrPOD releases ethene from ACC in the presence of bromide but not chloride (data not shown). With CTP, very low ethene yields (between 1 and 2% of the HrPOD reaction with bro-

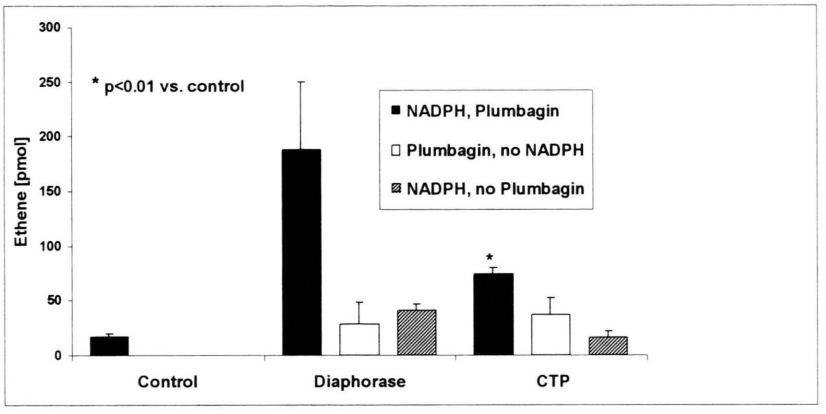

Fig. 5. Test of CTP for NAD(P)H oxidoreductase activity.
The test system is based on ethene release from KMB driven by redoxcycling of the naphthoquinone, plumbagin catalysed by diaphorase (pig-heart 5U; one unit is defined as oxidizing 1 μmol of NADH per min at pH 7.5 at 25 °C) or CTP (0.36 mg protein/ ml). The test system contained in 2 ml: 0.1 M Tris buffer pH 7.4, 1.5 mM KMB, 20 μM plumbagin, 0.5 mM NADPH and 5 U diaphorase (pig-heart) or 0.6 mg CTP-protein. The reaction was conducted for 30 min at 37 °C.

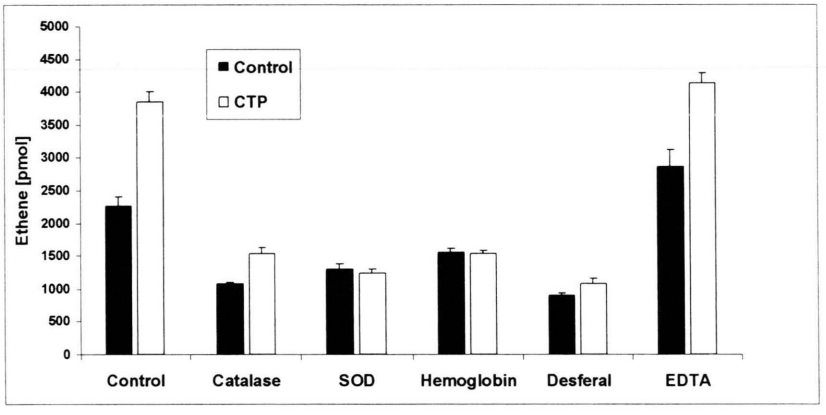

Fig. 6. Ethene release from KMB driven by SIN-1: Effects of CTP, SOD, catalase, hemoglobin, desferal and EDTA.
The reaction mixture contained in 2 ml: 0.1 M Tris buffer pH 7.4, 1.5 mM KMB, 50 μM SIN-1, 0.85 mg CTP-protein, 100 U catalase or SOD, 50 μM hemoglobin, 0.5 mM EDTA or 76 μM desferal.
The reaction was conducted for 30 min at 37 °C.

mide) could be measured. Since this activity is very low, corresponding to approx. 0.6 units HR-POD per mg CTP (data not shown), there is a high uncertainty in assuming that CTP is POD-active.

Catalase activity

As already reported by Gsell *et al.*. (1995), preparations from different regions of the brains, contain both SOD and catalase activity. As shown in Fig. 7 in comparison to commercially available catalase CTP contains approx. 20% of the activity of the added commercial catalase. If we calculate this activity on the basis of the added CTP, approx. 4 catalatic enzyme units correspond to 1 mg of CTP-protein.

Conclusions

CTP contain redox-modulatory properties:

1. Stimulation of oxidative reactions can be shown for superoxide-generating systems such as XOD or Sin-1. This property is most probably due to bound iron ions, not identical to hemoglobin.

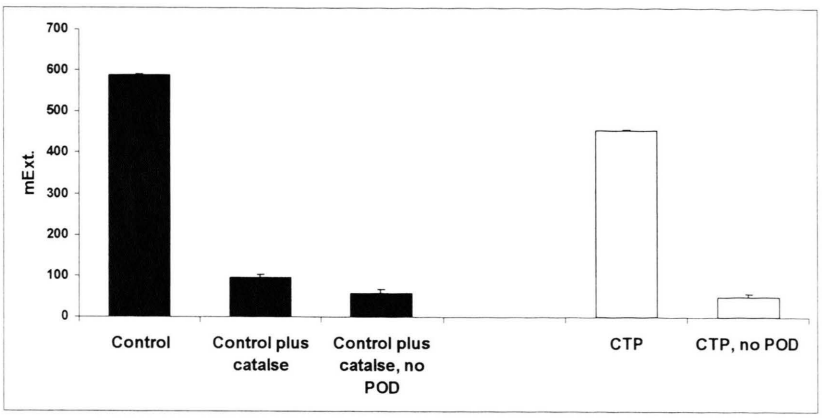

Fig. 7. Catalase activity of the CTP.
After incubation of 75 µM H_2O_2 with 25 U catalase or 1 mg CTP for 30 minutes, 7 mM 2-hydroxy-3,5- dichlorobenzosulfonate (HDBS), 0.5 mM 4-aminoantipyrine (4-AA) and 25 U POD were added to the reaction mixture in order to quantify the residual H_2O_2 colorimetrically at 510 nm.

This reaction may have physiological significance since glutamate receptors exhibit oxidative destructions after prolonged excitation.

2. CTP posses NADPH oxidase properties in the presence of redox-cycling naphtho- or anthraquinons, such as plumbagin. This reaction may play a role after certain drug treatments or intoxications with compounds passing the blood brain barrier and acting as redox cofactors.

CTP furthermore may contain very weak peroxidase- (myeloperoxidase-) activity

3. In contrast to SIN-dependent oxidations which are stimulated by CTP, ONOOH-dependent reactions are inhibited. In addition CTP contain 4 U catalase per mg protein.

Although the overall physiological importance of this redox-modulations are unknown one might speculate on possible protective functions of ACC which binds to the receptors and KMB, another methionine derivative derived via transamination.

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