Effects of hydroperoxides on contractile reactivity and free radical production of porcine brain arteries

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Abstract. Hydroperoxide-induced oxidative stress is assumed to be involved in vasospasm of cerebral arteries after subarachnoid hemorrhage. In order to study underlying mechanisms the effects of H_2O_2 and *tert* butylhydroperoxide on contractile functions as well as on free radical production of anterior cerebral artery from pig were investigated. The hydroperoxides increased in a similar dose-dependent arterial contraction, however, the underlying mechanisms involve impairment of endothelial-dependent relaxation as well as of smooth muscle contractile function as shown by experiments with inhibited endothelial NO-synthesis. Determination of lucigenine detectable O_2^--release showed rather low values with both hydroperoxides, whereas, in comparison, the unspecific luminol-dependent chemi-luminescence was increased up to 1000-fold, especially by H_2O_2. In conclusion it was shown that application of the hydroperoxides resulted in increased contraction of cerebral arteries, yet that the underlying mechanisms altering function of endothelial and smooth muscle cells need further exploration.

Key words: Brain arteries — Oxidative stress — Arterial contraction — Endothelium-dependent relaxation — Free radical production

Introduction

Cerebral vasospasm after subarachnoid hemorrhage (SAH) is a severe complication often responsible for death or strong impairment of brain function of the patients (Janjua and Mayer 2003). Its pathogenesis is still not completely understood, but evidence is provided that the underlying mechanisms are caused or at least accompanied by oxidative stress (Sonobe and Suzuki 1978; Gutteridge 1986; Braughler and Hall 1989; Gaetani et al. 1998; Janjua and Mayer 2003; Liu et al. 2007). Besides the activation of leukocytes producing reactive oxygen species (mainly O_2^-) *via* the NADPH-oxidase reaction, a dominant source of superoxide anion (O_2^-) is also found in the autoxidation of hemoglobin present in the subarachnoid space. Since by interaction with transition metals like Fe^{2+} the Fenton reaction will occur, different reactive species and degradation products like OH radical, H_2O_2, lipid peroxides, malondialdehyde will be generated in the extravasal compartment (Sano et al. 1980; Polodori et al. 1997; Kaynar et al. 2005). These reactive compounds were shown to induce contraction in different arteries of various species (Pelaez et al. 2000; Gao and Lee 2001; Zhang 2007).

Other explanations for vasospasm derive from the fact that O_2^- or ferrous hemoglobin can react with or bind to NO thus reducing the bioavailability of this endothelial relaxing factor (Pluta et al. 2001; Treggiari-Venzi et al. 2001; Janjua and Mayer 2003).

The importance of oxidative stress for cerebral vasospasm is supported also from the finding that pharmacological antioxidants or upregulation of antioxidative enzymes reduce the occurrence of peroxidative degradation products and vasospasm (Treggiari-Venzi et al. 2001; McGirt et al. 2002; Endo et al. 2007).

In order to characterize further mechanisms related to cerebral vasospasm we were interested to study the effects of oxidative stress induced by H_2O_2 and *tert* butylhydroperoxide (*tert* BHP) on serotonin (5HT)-stimulated contractions and free radical productions of anterior cerebral artery from pig brain.

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Materials and Methods

Arterial samples

Pig brains were delivered from a local slaugther house within 3 h after death of the animals. The anterior part of the cerebral artery was excised under an operation microscope, and cleaned from blood and adhering tissue. Ring preparations (4 mm long) were dissected and used for contractions measurements and detection of free radicals, respectively.

Contraction measurement

The arterial rings were mounted in a thermostated (37°C) organ bath perfused by Tyrode solution, which allowed registration of contraction forces under isometric conditions as already described (Wagner et al. 2000). After prestretching the sample to 3 mN and equilibration for at least 30 min until a stable tone was achieved, contractions were evoked by application of 5HT (10 µmol/l) to the Tyrode solution.

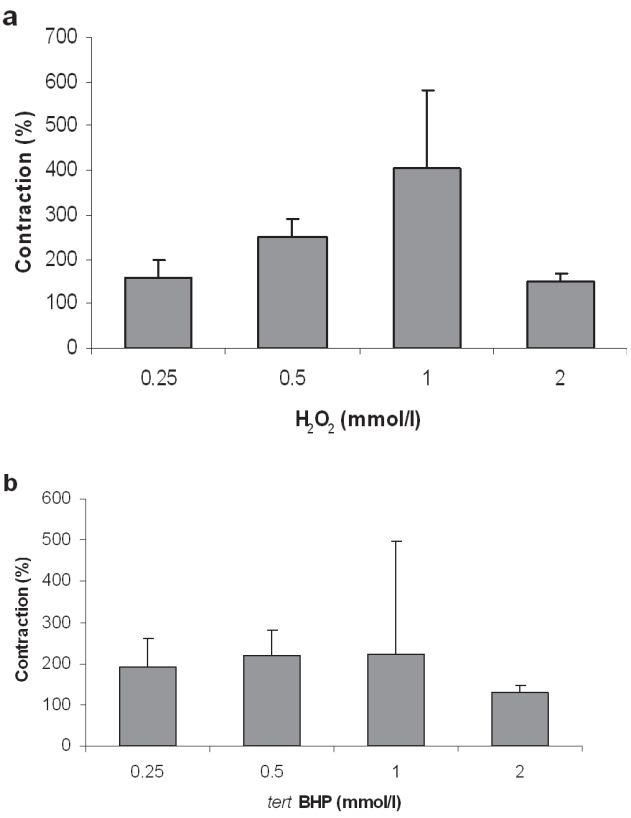

Figure 1. Effects of H_2O_2 (a) and *tert* butylhydroperoxide (*tert* BHP) (b) on serotonin (5HT)-induced contraction: dose response relation. The bars represent mean values ± SD, (n = 4–6). Absolute force of the control contraction induced by 10 µmol/l 5HT = 2.8 ±1.2 mN.

Generally, this type of vessels react under these conditions with a long lasting tonic contraction, the maximal force was evaluated. After wash out of the agonist, the stimulation with 5HT was repeated in the presence of H_2O_2 or *tert* BHP (final concentrations 0.25, 0.5, 1.0, 2.0 mmol/l). Each peroxide application was preceded by a control stimulation, a maximum of 3 applications were performed per arterial sample. The evaluated forces were related to the individual maximal 5HT-induced contraction of the corresponding specimen.

In order to estimate endothelial-dependent effects, in a second series of experiments 5HT was applied in the presence of 50 µmol/l nitroarginine (NA) (this antagonist was preperfused 10 min before 5HT-stimulation). Again, after wash out of 5HT, the stimulation was repeated with H_2O_2 or *tert* BHP (0.25 mmol/l) in the presence of NA (50 µmol/l). The effects on the contraction forces were evaluated and expressed in relation to the control contraction.

Measurement of free radical production

In order to characterize oxidative stress induced by the hydroperoxides, free radical generation was measured by chemiluminescence in a Biolumat 9600 (Berthold, Wildbad, Germany). Luminol and lucigenine were used as radical sensitive dyes and applied in separate incubation experiments. The arterial samples as prepared for the contraction experiments were incubated in 500 µl of Tyrode solution in the precence of the dye (0.1 mmol/l), and basal chemiluminescence was measured for 10 min. Thereafter H_2O_2 or *tert* BHP were added (final concentrations 0.05, 0.125, 0.25, 0.5, 1.0 mmol/l) and chemiluminescence was measured for another 10 min. Chemiluminescence, expressed as integrated counts per 10 min, was related to the dry weigth of the sample. The spontaneous chemiluminescence of the peroxide with the dyes was respectively considered.

Results

Effects of H_2O_2 and tert BHP in intact arteries

Fig. 1 shows that both hydroperoxides evoked similar dose-dependent effects on 5HT-induced contractions in the brain arteries: in concentrations below 1 mmol/l the standard contraction was increased up to 3–4-fold by H_2O_2 and up to 2-fold by *tert* BHP. At 2 mmol/l, inhibition was found with both peroxides. The further experiments were conducted with peroxide concentration of 0.25 mmol/l.

Effects of endothelium

When the preparations of anterior cerebral artery were stimulated by 5HT, no acetylcholine-induced, endothelium-

dependent vasorelaxation could be found (experiments were not described). However, effects of endothelium could be demonstrated when the stimulation occured in the precence of 50 µmol/l NA: under this condition a 3–4-fold increase in contraction force was found, showing that 5HT stimulates both smooth muscle contraction and endothelial NO production (Fig. 2, left panel).

In contrast, when the peroxides were applied in the presence of NA, 5HT induced a significantly decreased contraction force when compared with the NA effect without oxidative stress (Fig. 2, middle and right panel). Additionally, the figure shows again similar effects of both peroxides on arterial contractility under these conditions. These results suggest that peroxide-induced oxidative stress can modulate the contraction of brain arteries by inhibition of both smooth muscle contractility as well as endothelium-dependent relaxation.

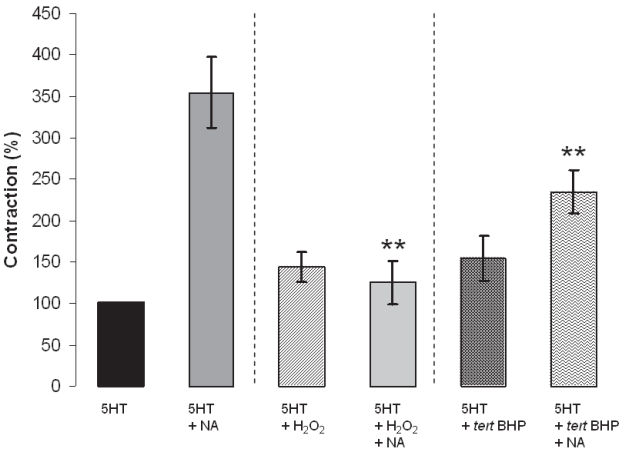

Figure 2. Effects of H_2O_2 and *tert* butylhydroperoxide (*tert* BHP) on serotonin (5HT)-induced contraction: importance of endothelium. Nitroarginine (NA) 50 µmol/l, H_2O_2 and *tert* BHP 250 µmol/l. The bars represent mean values ± SD, (n = 4–6), ** $p < 0.01$ compared with 5HT+NA, t-test.

Effects on free radical production

The consequences of hydroperoxide evoked oxidative stress on generation of free radicals were determined with the chemiluminescence dyes luminol and lucigenine. Table 1 shows that generally, the chemiluminescence is very much higher with H_2O_2, especially, when luminol is used as unspecific indicator of free radicals. However, the O_2^- specific dye lucigenine indicates similar chemiluminescence with H_2O_2 or *tert* BHP suggesting that O_2^- are formed with similar rates.

Discussion

Since 5HT is asssumed to be involved in vasospasm after SAH (Janjua and Mayer 2003) and since it evoked reproducible contractions in cerebral artery *in vitro,* we were interested in effects of oxidative stress induced by H_2O_2 and *tert* BHP on 5HT-induced contractions and free radical production.

Regarding the cellular metabolism of both peroxides, it is obvious that they induce different forms of oxidative stress: H_2O_2 is decomposed very effectively by catalase, but is a partner of the Fenton reaction to produce OH and different other radicals, *tert* BHP is mainly a substrate of glutathione peroxidase thus altering especially the sulfide-disulfide status of the cell.

Nevertheless, in the contraction experiments both peroxides revealed similar effects with similar dose response relations. The increase in contraction force, as seen in peroxide concentrations up to 1 mmol/l, could be a result of a direct stimulating effect on contractile apparatous of smooth muscle, e.g. by increasing cytoplasmic Ca^{2+}. Similar effects of H_2O_2 were also shown in other arterial preparations from various species and different parts of the arterial tree (Gao and Lee 2001; Blumenstein 2004; Gil-Longo and Gonzalez-Vazquez 2005; Zhang 2007). Another explanation

Table 1. Effects of H_2O_2 and *tert* BHP on reactive oxygen species production

	Luminol		Lucigenin	
Peroxide concentration (mmol/l)	H_2O_2 (counts per 10 min/mg)	*tert* BHP (counts per 10 min/mg)	H_2O_2 (counts per 10 min/mg)	*tert* BHP (counts per 10 min/mg)
0.05	300 ± 105	35 ± 20	220 ± 85	450 ± 75
0.125	19735 ± 6330	60 ± 40	780 ± 420	435 ± 185
0.25	164106 ± 42780	155 ± 95	1800 ± 190	930 ± 85
0.5	230900 ± 95320	1600 ± 1250	5825 ± 530	2890 ± 355
1.0	420870 ± 137680	8720 ± 5210	4260 ± 1590	3100 ± 1620

The values are mean values ± SD, n = 3–4.

for contraction stimulation could be that hydroperoxides inhibit endothelial-induced relaxation, thus increasing the resulting contraction force as also suggested for cerebral vasospams (Pluta et al 2001; Treggiari-Venzi et al. 2001; Janjua and Mayer 2003).

Yet, the results presented here show even more complex interactions: under blockade of endothelial NO synthesis by NA, the response to 5HT was 3–4-fold increased, indicating that the reaction of the intact vessel to this agonist was a moderate contraction as the sum of a strong stimulation of smooth muscle to contract and an additional stimulation of endothelial cells to produce the relaxing factor NO. When additionally oxidative stress was applied, the contraction stimulating effect of 5HT was significantly diminished. This means that in brain arteries rather low concentrations of hydroperoxides (0.25 mmol/l) can also lead to inhibited contractility, which is in contrast to all other samples of different arteries we investigated (Blumenstein 2004; Zhang 2007). Wether this mechanism is mediated by effects on ion channels, second messengers, or on the contractile proteins directly remains to be elucidated.

Under the pathological conditions of SAH, one can therefore assume that oxidative stress can induce different pathogenetic mechanisms, yet, maintenance or recovery of endothelial function is important in any case.

It is interesting to note that in coronary arterioles from pig and aorta from rat various signaling pathways were described to be effected by H_2O_2 (Thengchaisri and Kuo 2003; Gil-Longo and Gonzalez-Vazquez 2005). These include endothelium-dependent and -independent mechanisms which are related to the activation of cyclooxygenase as well as of potassium channels. Peroxide-induced hyperpolarisation of the membrane potential could be a direct relaxant effect in smooth muscle. In brain vessels these mechanisms have to be established by additional studies.

The different metabolism of the both hydroperoxides applied is clearly reflectd by the results of the free radical measurements. Using dye-enhanced chemiluminescence for their detection, the results showed that amount and nature of the generated free radicals are quite different using H_2O_2 and *tert* BHP, a phenomen also found e.g. in mouse aorta (Zhang 2007). Especially with H_2O_2 and the unspecific dye luminol, a high rate of free radical production was detectable, probably due to chain reactions of Fenton type. Using the O_2^- specific dye lucigenine, similar low rates were found with both peroxides. This could be due to a peroxide-dependent stimulation of NADPH oxidases present in the vascular wall generating O_2^- (Li et al. 2007). These results suggest that not the total rate of free radicals but perhaps the endogenous O_2^- production is responsible for hydroperoxide-induced effect on contractility of a. cerebri anterior.

In conclusion, the study shows that in 5HT-stimulated a. cerebri anterior there is an explicit oposing balance between smooth muscle contraction and endothelial-dependent relaxation.

Oxidative stress as induced by hydroperoxides can disturb this balance by inhibiting the endothelial function and also by impairment of smooth muscle contraction. Severe oxidative stress may induce smooth muscle relaxation probably by cellular damage of smooth muscle (Gil-Longo and Gonzalez-Vazquez 2005), however, under moderate conditions enhanced contractions will result. Therefore maintenance of endothelial function seems to be important to prevent or decrease vasospasm in cerebral arteries.

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