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PEROXIDE INDUCED ACTIVATION OF GLYCOGEN PHOSPHORYLASE A ACTIVITY IN VASCULAR SMOOTH MUSCLE.

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SUMMARY: The influence of oxidizing agents on the activity of glycogen phosphorylase in arterial smooth muscle was investigated in vitro. Parallel to an increase in the GSSG/GSH ratio, a strong activation of phosphorylase a was found, whereas the total activity of phosphorylase a+b was only slightly increased. Several possible mechanisms which can explain this finding are discussed.

INTRODUCTION: Similar to several other tissues, the activity of glycogen phosphorylase (EC 2.4.1.1) in arterial smooth muscle is known to be regulated by various factors which use Ca^{2+}-ions as a mediator for activating phosphorylase b kinase (EC 2.7.1.38) (1). Alternatively it is possible that the dephosphorylation of phosphorylase a to the b-form is regulated by influencing the activity of the phosphorylase phosphatase (EC 3.1.3.17). Recently it was shown that this enzyme isolated from liver is reversibly inhibited by oxidized glutathione via mixed disulfide formation (2,3). Thus, under the conditions of an increased degree of glutathione oxidation, one could expect increased levels of phosphorylase a due to the decreased dephosphorylation reaction.

In this study experiments were described in which aortic tissue was incubated in the presence of the oxidizing agents t-butylhydroperoxide and diamide and the effects on phosphorylase activity and glutathione redox state were investigated. The results show that in vascular smooth muscle, peroxidative stress increases the GSSG/GSH ratio as well as the phosphorylase a/b ratio.

MATERIAL and METHODS: Aortas were obtained from 8 male New Zealand rabbits weighing 2 - 2.5 kg, which had been maintained on

Abbreviations: GSH, GSSG, glutathione, reduced and oxidized; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; t-BHP, tert. butylhydroperoxide.

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a standard chow at 50 g/kg per day and water, ad libitum. The animals were anaestethized with nembutal (Abbott, S.A., Ottignies Belgium) (40 mg/kg b.wt., i.v.), and the thoracic segment of the aorta descendens was excised, and freed from fat, blood and connective tissue. During the preparation the tissue was immersed in buffered, air equilibrated, ice cold Tyrode solution (composition in mM: NaCl 118; KCl 5.83; NaH_2PO_4 1.54; $MgSO_4$ 1.19; $NaHCO_3$ 11.9; $CaCl_2$ 2.53; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) 20; pH 7.4). Afterwards the vessel was cut in rings of about 1 cm length and used for the following experiments.

Samples were incubated in a 37°C waterbath with agitation (60/min) for intervals of up to 30 min in the presence of t-butylhydroperoxide (Schuchardt, München, FRG) (2 mM) or of diamide (5 mM, Sigma, München, FRG) dissolved in Tyrode solution. Peroxide was also applied in saline (150 mM). After the peroxide incubation each ring was divided longitudinally in two parts which were used for the determination of phosphorylase and GSSG/GSH respectively. As controls segments were used which were analysed either immediately after preparation or after the same incubation periods in normal Tyrode solution.

For the determination of the activity of phosphorylase, fresh tissue (10-20 mg w.wt.) was minced with scissors, and homogenized with ice cold triethanolamine buffer (0.4 ml, 0.1 M, pH 7.0) containing NaF (20 mM) and EDTA (1 mM). Following centrifugation (12 000 g, 10 min, 4°C) phosphorylase a was determined in the supernatant essentially according to Cori's method, in which the liberation of inorganic phosphate from glucose-1-phosphate is measured in the synthesising reaction. Keeping the substrate concentrations constant, the incubation volume was reduced to 0.4 ml using 0.05 ml of the tissue extract. Free phosphate was determined in aliquots (0.01 ml) taken at 0, 30, 60, 90 min, after starting the reaction (5). The total amount of enzyme activity (phosphorylase a+b) was determined in the presence of AMP (2 mM). In each phosphorylase assay, controls were performed without the addition of glycogen, showing that the glycogen-independent liberation of phosphate from glucose-1-phosphate is negligible under these conditions, but that there are substantial amounts of phosphate produced from the nucleotide. Enzyme activities were calculated from the time-dependent increase of the phosphate concentrations taking into account the corresponding controls. Protein was determined in the supernatant after deproteinizing an aliquot by perchloric acid and redissolving the protein in 1N NaOH (6). This procedure was necessary due to the high blank value caused by triethanolamine in the protein determination.

Due to the interference of diamide with the assay for glutathione, the oxidation state of glutathione was only determined after the peroxide incubation. The tissue samples were lyophilized, weighed, and soluble total glutathione and GSSG were determined as recently described (7). The results are expressed as "mol GSSG/100 mol GSH".

Hepes was obtained from Serva, Heidelberg, FRG, AMP, glucose-1-phosphate, glycogen and triethanolamine were from Boehringer, Mannheim, FRG, and all other substances were p.A. grade chemicals from Merck, Darmstadt, FRG.

RESULTS: Table 1 summarizes results of the incubation experiments and shows that the activity of phosphorylase a increased about

Table 1.: Effect of t-BHP and diamide on the activity of glycogen phosphorylase a (determined in the absence of AMP) and the total phosphorylase activity (determined in the presence of 2 mM AMP) in aortic smooth muscle of rabbit.

Incubation condition	phosphorylase a	total phosphorylase
immediately after preparation (n=5)*	5.0 ± 3.3	114.8 ± 33.3
normal Tyrode solution (n=4)*	5.5 ± 3.6	119.0 ± 18.5
t-BHP (2 mM) in Tyrode (n=6)*	39.2 ± 15.5	144.0 ± 21.5
diamide (5 mM) in Tyrode**	31.2	122
t-BHP (2 mM) in 150 mM saline (n=3)*	42.5 ± 10.2	130.5 ± 25.4

Aortic tissue samples were homogenized either immediately after preparation or after 30 min of incubation at 37°C under the conditions indicated. Phosphorylase activity was determined in the supernatant measuring phosphate-liberation from glucose-1-phosphate in the glycogen synthesising reaction.
The values (given as mU/mg protein) represent either (*) mean values ± S.D. or (**) the results of a single experiment. For further details, see methods.

7 fold when the aortic rings were incubated under oxidizing conditions. Qualitatively there was no difference in the stimulating effect using either t-butylhydroperoxide or diamide. Furthermore, the data show that the peroxide-dependent stimulation could also be observed using an incubation solution free of calcium ions. The total activity of phosphorylase (a+b) as determined in the presence of AMP reveals only a slight increase under the oxidizing conditions.

The time dependency of the peroxide action on the GSSG/GSH ratio and on phosphorylase a activity is shown in table 2. In comparison with the peroxide free incubated controls, both parameters were increased during the incubation time. The increase of the GSSG/GSH ratio indicates that the aortic peroxide detoxification *via* glutathione peroxidase is limited by the capacity of glutathione disulfide reductase.

DISCUSSION: t-Butylhydroperoxide and diamide are well established agents for experimentally induced, reversible alterations in the GSSG/GSH system of intact tissue (8-10). In aortic tissue the reversibility of the peroxide action on the GSSG/GSH-ratio has been previously reported (11).

Table 2.: Time dependency of t-BHP action on phosphorylase a activity and GSSG/GSH ratio in aortic smooth muscle of rabbit.

Time (min)	phosphorylase a	GSSG/GSH
0	3.3	2.8
with t-BHP 2 mM		
10	7.5	4.2
20	13.0	7.9
30	28.5	12.5
without t-BHP		
30	4.0	3.5

Aortic tissue samples were analysed immediately after preparation or after incubation with/without t-butylhydroperoxide (2 mM) at the time intervals indicated. Each sample was divided in two parts, one was used for the determination of phosphorylase a (without AMP), the other for the evaluation of GSSG/GSH ratio. The experiment was performed twice, the results of one differing not more than 25% from the other are reported. Phosphorylase activity is given in mU/mg protein, the redox-state of soluble glutathione in mol GSSG/100 mol GSH. For further details see methods.

It is obvious from this study that under oxidizing conditions, as reflected by an increased intracellular GSSG/GSH ratio, a stimulation of phosphorylase a activity is found in smooth muscle of rabbit aorta. Besides the possibility of unknown interactions, the following mechanisms come into consideration:

1) The activation could be an apparent one, if, in the enzymatic assay, the endogenous AMP concentration is increased. Assuming that during the incubation period the sum of the total ATP and ADP pools, about 1 mM for rabbit aorta (12), is converted to AMP, the final concentration change of AMP in the test tube would not exceed 0.02 mM, which is too low for a seven fold activation of phosphorylase b in the test of phosphorylase a. According to Namm (1) a 9 fold activation of rabbit aortic phosphorylase activity is found by increasing AMP from zero to 1 mM in the assay.

2) Due to the action of the agents themselves or the decreased reduction potential of the cells, the calcium entry or intracellular release could be increased, activating phosphorylase b kinase. However, since the stimulation is still found in Ca^{2+}-free solution, only membrane bound or intracellular calcium ion stores are likely to play a role.

3) According to the above mentioned hypothesis, the results could be regarded in terms of an inhibition of phosphorylase phosphatase by the increased GSSG/GSH ratio, thus depressing the

turnover of phosphorylase a to b and increasing the level of phosphorylase a. Duyckaerts et al (13), however, reported that the disulfide cystamine, which is described by Usami (3) as inhibiting phosphorylase phosphatase to an even greater extent than GSSG, increases phosphorylase a activity by stimulating the phosphorylase b kinase reaction.

At the moment it is not possible to decide between the two alternative mechanisms: stimulation of the kinase reaction caused by the oxidative stress, probably as a result of liberation of endogenous calcium, or inhibition of phosphorylase a degradation by inactivation of the phosphatase caused by the increased GSSG/GSH ratio.

Although the exact mechanism of stimulation of glycogen phosphorylase by oxidative stress remains to be elucidated, this finding fits in well with the observation (14) that glycogenolysis in liver is increased during perfusion with t-butylhydroperoxide.

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