The influence of free radicals and other reactive oxygen species on pharmacological actions in the cardiovascular system

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Summary

Chapter 1

This chapter is dealing with the chemical nature and general aspects of free radicals and other reactive oxygen species (ROS), and possible sources of ROS in vivo. Since most ROS are very reactive, they will readily react with important cellular components (such as lipids, proteins and nucleic acids) and possibly alter various cellular processes. Most organisms have developed antioxidant systems to protect their cells against the deleterious effects of ROS. These defences can be enzymatic (superoxide dismutase, glutathion peroxidase system and catalase) as well as non-enzymatic such as for instance antioxidant vitamins. An increased production of ROS can overwhelm the local antioxidant system, thus leading to a situation termed "oxidative stress" which may provoke oxidative damage. A variety of disease states with direct or indirect cardiovascular implications (for instance ischaemia/reperfusion, atherosclerosis, diabetes and congestive heart failure), in which ROS may play a role, are discussed. Since ROS are implicated in these disease states, clinical trials have addressed the question whether antioxidant therapy may be beneficial in these diseases. However, the results obtained so far are disappointing and not clear. Furthermore, an overview is given of possible cellular targets of ROS that may explain the deleterious effects of ROS on the cardiovascular system.

Chapter 2

The effects of ROS, induced by electrolysis of a physiological salt solution, on various inotropic responses in isolated rat left atria were studied. Evidence for the generation of hydroxyl radicals was obtained from an appropriate fluorimetric assay. The amount of ROS produced by electrolysis of the medium proved current-dependent. Exposure of isolated rat left atria to the medium which had been subjected to electrolysis caused a current-dependent decrease in contractile force. Oxidative stress, as a result of the electrolysis of the medium, caused blunted inotropic responses to extra cellular Ca^{2+}, sodium withdrawal and lowering of stimulation frequency. The response to the β-adrenoceptor agonist isoprenaline was diminished in atria subjected to oxidative stress and led to a rightward shift of the concentration respons curves (pD_2 contol group: 7.56 ± 0.10 vs. 6.77 ± 0.11 electrolysis group). In addition, the inotropic responses to forskolin (pD_2 control group: 6.17 ± 0.12 vs. < 4.5 electrolysis group) and dibutyryl cAMP proved blunted as well. Measurement of the adenylyl cyclase activity revealed that ROS attenuated the basal and forskolin stimulated activity of the adenylyl cyclase. DMSO, a well known hydroxyl radical scavenger, was able to abolish the free radical-induced decrease in the response to isoprenaline. Surprisingly, α-adrenoceptor stimulation in atria subjected to

electrolysis-generated ROS led to a rapid decrease in contractile force. DMSO was unable to counteract the negative intropic effect of methoxamine in atria subjected to oxidative stress. This negative inotropic response to α-adrenoceptor stimulation in atria subjected to electrolyzed medium is unlikely the direct result of phospholipase C or protein kinase C activation. Angiotensin II (which stimulates PLC as well) did not show an inotropic effect and chelerythrine (a PKC inhibitor) was unable to counteract the negative inotropic effect after α-adrenoceptor stimulation.

From this study we can conclude that free radicals alter responses to various inotropic stimuli. These alterations may be the result of injured contractile elements, transporter molecules and molecules involved in signal transduction. α_1-Adrenoceptor stimulation after oxidative stress leads to a PLC and PKC-independent negative inotropic effect.

Chapter 3

This study was designed to investigate the mechanism(s) of the negative inotropic effects of α_1-adrenoceptor agonists observed in isolated rat left atria after exposure to ROS. Ouabain and calphostin C were used in contraction experiments to block the sodium pump and protein kinase C. Methoxamine-induced phospholipase C and Na^+/K^+ ATPase activities were measured. Methoxamine (300 μM) increased contractile force by 1.6 ± 0.2 mN in control atria but decreased contractile force in electrolysis-treated atria by 2.0 ± 0.1 mN, as determined 10 min after methoxamine addition. In contrast, the positive inotropic effects of endothelin-1 (30 nM) and isoprenaline (10 μM) were reduced from 2.6 ± 0.3 to 1.3 ± 0.1 mN and from 2.6 ± 0.3 to 1.7 ± 0.2 mN, respectively, by electrolysis treatment, but not converted into a negative inotropic action. In an inositol phosphate assay we observed that the stimulation of phospholipase C by methoxamine was attenuated by electrolysis when the (electrolyzed) medium from the organ bath was used, but the phospholipase C responses were restored by the use of fresh medium. However, medium refreshment did not counteract the negative inotropic effect of methoxamine. Accordingly, the negative inotropic effect of methoxamine is not directly related to the impaired phospholipase C responses seen in atria subjected to electrolysis.

Ouabain and the protein kinase C inhibitor calphostin C, completely prevented the negative inotropic effect of 300 μM methoxamine in electrolysis-treated atria. Measurement of the Na^+/K^+ ATPase activity, revealed that in control atria, α_1-adrenoceptor stimulation with 300 μM methoxamine, decreased the Na^+/K^+ ATPase activity by 14.4 ± 7.7%. In contrast, methoxamine increased the Na^+/K^+ ATPase activity by 48.8 ± 8.9% in electrolysis-treated atria. Interestingly, this

increase in Na^+/K^+ ATPase activity was completely counteracted by calphostin C (1.4 ± 0.1% over basal).

These results indicate that the negative inotropic effects of α_1-adrenoceptor agonists, observed in isolated rat left atria exposed to ROS, is likely to be caused by protein kinase C mediated phosphorylation and subsequent activation of the Na^+/K^+ ATPase.

Chapter 4

In the study described in this chapter the influence of hydrogen peroxide on the rat heart was investigated. Hydrogen peroxide (600 µM for 9 min) induced a pronounced increase in left ventricular pressure (LVP) which reached a stable maximum 30 min after the H_2O_2 infusion period. The thromboxane A_2 antagonist SQ29548 was unable to counteract this increase in LVP. Pretreatment of the hearts with the dual cyclooxygenase/lipoxygenase inhibitor meclofenamate resulted in a pronounced increase in left ventricular end diastolic pressure, indicating protective effects of eicosanoids. In hearts exposed to hydrogen peroxide the inotropic responses to α_1-adrenoceptor stimulation were completely diminished, whereas the responses to β-adrenoceptor stimulation were only blunted. Ouabain and calphostin C were unable to counteract the disappearance of the α_1-adrenoceptor responses. These data indicate that the disappearance of the α_1-adrenoceptor responses is caused by a different mechanism than that observed in the isolated atria.

Chapter 5

The aim of the present study was to investigate the influence of reactive oxygen species (ROS) on the contractile responses of rat isolated left atria to muscarinic receptor stimulation. ROS were generated by means of electrolysis (30mA, 75 s). Twenty minutes after the electrolysis period the electrically paced atria (3 Hz) were stimulated with the adenylyl cyclase activator forskolin (1 µM). Subsequently, cumulative acetylcholine concentration-response curves were constructed (0.01 nM - 10 µM). In addition, phosphoinositide turnover and adenylyl cyclase activity under basal and stimulated conditions were measured. For these biochemical experiments we used the stable acetylcholine analogue carbachol. The atria exposed to reactive oxygen species were influenced more potently (pD_2 control: 6.2 ± 0.1 vs. 7.1 ± 0.1 for electrolysis treated atria, $P<0.05$) and more effectively (E_{max} control 40% vs. 90% reduction of the initial amplitude, $P<0.05$) by acetycholine. The basal (-40% vs. control) as well as the carbachol-stimulated (-85% vs. control) inositolphosphate formation was reduced in atria exposed to ROS. The

basal adenylyl cyclase activity was identical in both groups but carbachol-stimulation induced a more pronounced reduction in adenylyl cyclase activity in the electrolysis treated atria.
Accordingly, we may conclude that ROS enhance the negative inotropic response of isolated rat atria to acetylcholine by both a reduction of the positive (inositide turnover) and increase of the negative (adenylyl cyclase-inhibition) inotropic components of cardiac M_2-muscarinic receptor signal transduction cascade.

Chapter 6

We have investigated a possible role of mitogen-activated protein kinases (MAPK) in the contractile effects of the α_1-adrenoceptor agonist methoxamine and endothelin-1 in isolated rat left atria and thoracic aorta. Endothelin-1 (50 nM) and, to a lesser extent, methoxamine (300 μM) activated the ERK and p38 isoforms of MAPK as determined by immunoblotting with epitope-specific anti-phosphotyrosine antibodies. PD98059 (1 mM), an inhibitor of the ERK cascade, significantly reduced the inotropic responses to methoxamine and endothelin-1 by approximately 66% and 86%, respectively, but not that to isoprenaline (30 μM). None of these inotropic effects was inhibited by 2 μM of the p38 inhibitor SB203580. PD98059 also inhibited aortic constriction in response to methoxamine, and this was largely due to a reduced maximal response (-74%). The endothelin-1-induced aortic constriction was inhibited only marginally, and significant inhibition of endothelin-1 (-52%) by PD98059 was observed only in the presence of the calcium entry blocker verapamil (1 μM). PD98059 also inhibited the aortic contraction to 20 μM phorbol 12-myristate 13-acetate (-53%) but not that to KCl (40 mM). None of the responses in rat aorta was affected by SB203580.
We conclude that a PD98059-sensitive MAPK pathway is involved in the inotropic and vasoconstrictor actions of α_1-adrenoceptor agonists and endothelin-1. The activation of this pathway may be partially protein kinase C-dependent.

Chapter 7

Oxygen derived free radicals and other reactive oxygen species (ROS) are involved in a variety of disease states, which can have cardiac and vascular implications. The present study was performed to investigate the mechanism of ROS-induced vasoconstriction and the influence of ROS on the functional integrity of isolated rat thoracic aorta. ROS were generated by means of electrolysis (30 mA, during 0.5, 1, 2 and 3 min) of the organ bath fluid. ROS induced a transient (approximately 60 min) vasoconstriction and the maximally induced contraction was dependent on the duration of

electrolysis. Dimethyl sulfoxide diminished the ROS-induced vasoconstriction almost completely, indicating a major influence of hydroxyl radicals on contractility.

The dual cyclooxygenase/lipoxygenase inhibitor meclofenamate completely prevented the ROS-induced vasoconstriction. The PLA_2 inhibitor oleyloxyethyl phosphorylcholine was able to reduce the vasoconstriction elicited by ROS by approximately 70%. Conversely, the specific $cPLA_2$ inhibitor arachidonyl trifluoromethylketone proved ineffective in this respect. By using the specific mitogen-activated protein kinase (MAPK) kinase inhibitor PD98059 it was shown that the activation of extracellular-regulated kinase (ERK) MAPK contributes to the ROS-induced vasoconstriction.

The effects of ROS on the functional integrity of the aortae were investigated in particular with respect to receptor (α_1-adrenoceptor) and non receptor-mediated contractile responses (high potassium solution). In addition, the endothelium-dependent (methacholine) and endothelium-independent (sodium nitroprusside) vasorelaxation were investigated before and after ROS exposure. Electrolysis periods of 0.5 and 1 min. induced a modest leftward shift of the concentration response curves for the α_1-adrenoceptor agonist methoxamine. Longer electrolysis periods of 2 and 3 min. additionally decreased the maximal response to α_1-adrenoceptor stimulation.

Methacholine-induced vasorelaxation proved diminished in aortae subjected to electrolysis (0.5, 1, 2 and 3 min), whereas relaxation to sodium nitroprusside was nearly complete in all groups. KCl-induced contractions proved attenuated only after longer electrolysis periods of 2 and 3 min.

From these results we may conclude that ROS induce an eicosanoid and ERK MAPK-mediated vasoconstriction in isolated rat thoracic aorta. In addition, exposure to ROS leads to a deterioration of functional integrity characterized by endothelial dysfunction and decreased contractile function.