Pharmacological investigations on reactive oxygen species in the cardiovascular system

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Summary
Chapter 1

The introduction of the present thesis deals with the chemical characteristics of reactive oxygen species (ROS), their reactivity towards macromolecules and the role of ROS in the pathogenesis of various cardiovascular diseases. Usually, ROS are detoxified by an endogenous antioxidant defense system. However, when the amount of ROS exceeds the detoxifying capacity of such mechanisms, oxidative stress occurs. Enhanced levels of ROS cause damage to lipid membranes, DNA and proteins, leading to significant damage to tissues. Oxidative stress is a common feature in cardiovascular and metabolic diseases, especially in hypertension, heart failure, ischemia and diabetes. Based upon epidemiological surveys, it has been hypothesized that an antioxidant-rich diet (as used for instance in the Mediterranean area) could prevent or reduce cardiovascular morbidity and mortality. These promising effects were confirmed in various in vitro and animal models. Therefore, numerous intervention trials in humans have been conducted since, investigating antioxidants such as α-tocopherol and β-carotene. In contrast to the initial hypothesis, these antioxidants rarely decreased cardiovascular mortality. The majority of the intervention trials showed no antioxidant-derived benefit in patients with cardiovascular diseases. The rather conflicting outcomes between in vitro and animal studies on the one hand and intervention trials on the other may in part be caused by differences between human and experimental animal atherosclerosis. Furthermore, in the intervention trials the level of oxidative stress in these patients was not measured. When antioxidant treatment does not lower the markers of oxidative stress, a reduction of cardiovascular events cannot be expected. Only patients, in which elevated amounts of oxidative stress are established, should be enrolled in future intervention trials. The quantification of appropriate markers of oxidative stress may help to identify a subpopulation that might benefit from antioxidant supplementation.

Chapter 2

The aim of this study was to determine whether the antioxidant properties of thiol-containing compounds are related to their physico-chemical characteristics. For this purpose isolated rat left atria were subjected to electrolysis-induced oxidative stress. This type of oxidative stress has been shown to decrease the initial contractile force of isolated rat left atria. Accordingly, the protective properties of thiol-containing compounds were assessed by quantifying their effects on electrolysis-induced decline of contractile force. We studied the protective properties of six thiol-containing agents: penicillamine, mesna, N-acetylcysteine, mercaptopropionylglycine, captopril and glutathion. A possible correlation between the protective effect of these compounds (expressed as the log EC$_{50}$ values) and their lipophilicity (expressed as log P) and acidity of the thiol moieties (expressed as pK$_a$) was investigated. All thiol-containing compounds counteracted the ROS-induced phenomena in a concentration dependent manner. We established no positive correlation between the log EC$_{50}$ values and the log P values. Nor was there a correlation between pK$_a$ values and log EC$_{50}$ values. Even in an
analytical approach we could not find any relationship between the acidity and the antioxidant actions of the thiols.
Accordingly, in contrast to certain in vitro studies, the antioxidant properties of thiol-containing compounds do not appear to be dependent on their physico-chemical properties in a model of electrolysis-induced oxidative stress.

Chapter 3

Reactive oxygen species (ROS) are known to be involved in the pathogenesis and progression of various cardiovascular diseases. For therapeutics like carvedilol and captopril, used in the treatment of such diseases, antioxidant properties have been proposed to play a role in addition to the haemodynamic effects. It was the aim of the present study to assess whether ROS affect the molecular integrity and consequently the primary pharmacological actions of compounds with additional antioxidant properties.
Accordingly, carvedilol and captopril were exposed to ROS, generated by electrolysis, and analyzed by means of functional and chemical investigations.

For this purpose, rat thoracic aortic rings were incubated with either the β₂/α₁-adrenoceptor antagonist carvedilol, the α₁-adrenoceptor antagonist prazosin, the thiol-containing ACE-inhibitor captopril or lisinopril (an ACE-inhibitor without an antioxidant thiol moiety), respectively. Furthermore, isolated rat left atria were incubated with either carvedilol or with the β₁,₂-adrenoceptor antagonist timolol. Electrolysis was applied to the buffer solution in order to generate ROS. Subsequently, concentration response curves were constructed for angiotensin I, phenylephrine and isoprenaline in pre-treated thoracic aortic rings and isolated left atria, respectively. After exposure to oxidative stress, the α₁- and β-adrenoceptor blocking activity of carvedilol was significantly impaired when compared to control conditions. In contrast, the pharmacological effects of prazosin and timolol remained unaffected. ACE-inhibition by captopril was completely abolished after electrolysis, while the pharmacological action of lisinopril was only slightly reduced. In addition, a complete oxidative degradation of captopril and carvedilol could be demonstrated by using UV-Vis spectroscopy and HPLC/fluorospectroscopy, respectively.

From these results we conclude that therapeutics with additional radical scavenging properties may undergo a chemical modification due to ROS-exposure, which results in a loss of pharmacological activity.

Chapter 4

A well-known source of ROS are activated neutrophils, which can release superoxide radicals and hydrogen peroxide by membrane-bound NAD(P)H-oxidases. These ROS do not only destroy bacteria but may also affect mammalian tissue. In addition, hydrogen peroxide serves as a substrate for myeloperoxidase, an enzyme which is released by activated neutrophils during inflammatory processes as seen for instance in reperfusion injury and atherosclerosis.
Myeloperoxidase catalyzes the oxidation of chloride by hydrogen peroxide, yielding hypochlorite, an extremely potent oxidant.

The purpose of this study was to evaluate and compare the effects of hydrogen peroxide and hypochlorite on several receptor-dependent processes in isolated rat left atria and rat thoracic aorta.

In the presence of hypochlorite the positive inotropic response of \( \alpha_1 \)-adrenoceptor stimulation by methoxamine was converted into a negative inotropic response. In contrast, the positive inotropic effects of the \( \beta_1/\beta_2 \)-adrenoceptor agonist isoprenaline and endothelin-1 remained largely unaffected. The inversion of \( \alpha_1 \)-adrenoceptor-mediated inotropy was not observed in the presence of hydrogen peroxide. Furthermore, hydrogen peroxide did not influence the positive inotropic response to isoprenaline, however it completely abolished the positive inotropic effect of endothelin-1.

In addition, the effect of cardiac M3-receptor stimulation in the presence of hypochlorite and hydrogen peroxide was studied. Hypochlorite enhanced the negative inotropic response to acetylcholine, however, it did not amplify the concentration response curve for adenosine. Therefore we conclude that the hypochlorite-induced amplification is not common for all G-protein coupled receptors. In addition, we studied the effects of hypochlorite on the M3-receptor in the isolated rat portal vein. Since no enhancement was observed in the portal vein, we suggest that hypochlorite-induced oxidative stress selectively amplifies the response by M3-receptor stimulation, as shown in the isolated rat left atrium.

In the rat thoracic aorta, the endothelial function, evaluated by means of acetylcholine-induced vasodilation, was completely abolished in the presence of hypochlorite but was not affected by treatment with the same concentration of hydrogen peroxide.

From these data we conclude that the myeloperoxidase-driven formation of hypochlorite represents an amplification of the toxic properties of hydrogen peroxide, leading to substantial physiological alterations in cardiac and vascular tissue.

**Chapter 5**

As reported in chapter 4, exposure to hypochlorite-induced oxidative stress muscarinic led to an enhanced negative inotropic response to stimulation of M3-receptors in isolated rat left atria. This phenomenon was not observed after stimulation of the cardiac \( \Lambda_1 \)-receptor, which is, as the M3-receptor, coupled to Gproteins. Since also the contractile response to M3-receptor stimulation was not amplified by hypochlorite in the rat portal vein, we hypothesized a M3-receptor specificity of this hypochlorite-induced enhancement. The present study was performed in order to investigate whether the sympathoinhibitory response to presynaptic M3-receptor stimulation would also be modified after exposure to hypochlorite. Experiments were performed in the rat tail artery. In order to mimic sympathetic neurotransmission we applied electrical field stimulation (EFS), which increased the vascular tone frequency-dependently. EFS-induced vasoconstriction was attenuated by acetylcholine in a concentration dependent manner. In contrast to cardiac M3-receptors, hypochlorite did not amplify the sympathoinhibitory effect of acetylcholine in the rat tail artery.
The different response of neuronal and cardiac M$_2$-receptors to hypochlorite may be explained by the different G-protein subunits involved in the activation of the underlying signalling cascade.

Chapter 6

The activation of the mitogen-activated protein kinase (MAPK) pathway in the heart, by for instance $\alpha_1$-adrenoceptor agonists and endothelin-1, has been associated with cellular growth regulation. In this chapter we investigated a possible role of MAPK pathways in the inotropic and chronotropic effects of adrenoceptor and ET$_A$-receptor stimulation in isolated rat left and right atria. Inotropic as well as chronotropic responses were determined for the $\alpha_1$-adrenoceptor agonist methoxamine, the $\beta$-adrenoceptor agonist isoprenaline, the endothelin-1 and in the absence and presence of inhibitors of MAPK pathways. The MAPK kinase (MAPK$^{\text{mkk}}$) inhibitors PD98059 and U0126 significantly inhibited the inotropic responses to $\alpha_1$-adrenoceptor and ET$_A$-receptor stimulation, but not the chronotropic effects. U0126, but not PD98058 attenuated the inotropic response to isoprenaline. None of the aforementioned inotropic and chronotropic effects were inhibited by the MAPK$^{\text{p38}}$ inhibitor SB203580. We conclude that activation of the MAPK$^{\text{erk}}$ pathways is essential for the inotropic but not the chronotropic actions of adrenoceptor agonists and endothelin-1.

Chapter 7

ROS play a pathological role in cardiovascular diseases such as heart failure and hypertension. However, increasing evidence suggests that ROS can formed subsequent to the stimulation of various receptors, thereby functioning as second messengers. The objective of this study was to elucidate the role of endogenously generated ROS in the inotropic and chronotropic effects of $\alpha_1$- and $\beta_1$-adrenoceptor and ET$_A$-receptor stimulation in isolated rat atria. In addition, we investigated whether the MAPK$^{\text{erk}}$ pathway is involved in the ROS-provoked rise of contractile force. For this purpose hydrogen peroxide, which is known to serve as a second messenger, was applied as a contractile agent. Hydrogen peroxide readily crosses cell membranes thus allowing to mimick the effects of endogenous ROS on the contractile behaviour of the isolated rat left atrium. Pre-incubation of atria with EUK-8, a cell permeable superoxide dismutase- and catalase-mimetic, reduced the positive inotropic effect to $\alpha_1$-adrenoceptor and ET$_A$-receptor stimulation. The response to $\beta$-adrenoceptor stimulation remained unaffected by pre-treatment with EUK-8. Chronotropic effects were not altered by EUK-8. In contrast to the MAPK$^{\text{p38}}$ inhibitor SB203580, the two MAPK$^{\text{mkk}}$ inhibitors, PD98059 and U0126, significantly attenuated the positive inotropic response to hydrogen peroxide. We conclude that the inotropic responses to $\alpha_1$-adrenoceptor and ET$_A$-receptor stimulation are at least partially caused by endogenously formed ROS that subsequently may activate the MAPK$^{\text{erk}}$ pathway, thereby contributing to the increase in contractile force.