

**Role of oxidative stress in PKC- δ up-regulation and cardioprotection induced
by chronic intermittent hypoxia**

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Abstract

The aim was to determine whether increased oxidative stress during the adaptation to chronic intermittent hypoxia (CIH) plays a role in the induction of improved cardiac ischemic tolerance. Adult male Wistar rats were exposed to CIH in a hypobaric chamber (7000 m, 8 h/day, 5 days/wk, 24-30 exposures). Half of the animals received antioxidant N-acetylcysteine (NAC; 100 mg/kg) daily before the exposure; the remaining rats received saline. Controls were kept under normoxia and treated in a corresponding manner. One day after the last exposure (and/or NAC injection), anesthetized animals were subject to 20-min coronary artery occlusion and 3-h reperfusion for infarct size determination. In parallel subgroups, biochemical analyses of the left ventricular myocardium were performed. Adaptation to CIH reduced infarct size from 56.7 ± 4.5 % of the area at risk in the normoxic controls to 27.7 ± 4.9 %. NAC treatment decreased the infarct size in the controls to 42.0 ± 3.4 % but it abolished protection provided by CIH (to 41.1 ± 4.9 %). CIH decreased the reduced-to-oxidized glutathione ratio and increased the relative amount of protein kinase C (PKC) isoform- δ in the particulate fraction; NAC prevented these effects. The expression of PKC- ϵ was decreased by CIH and not affected by NAC. Activities of superoxide dismutase, catalase and glutathione peroxidase were affected by neither CIH nor NAC treatment. It is concluded that oxidative stress associated with CIH plays a role in the development of increased cardiac ischemic tolerance. The infarct size-limiting mechanism of CIH seems to involve the PKC- δ -dependent pathway but apparently not the increased capacity of major antioxidant enzymes.

Keywords:

ischemia-reperfusion, oxygen radicals; chronic hypoxia; infarct size; protein kinase C.

INTRODUCTION

Adaptation of rats to chronic intermittent hypoxia (CIH) increases cardiac tolerance to acute ischemia/reperfusion injury, as evidenced by reduced myocardial infarction, improved recovery of contractile function and limitation of ventricular arrhythmias (24, 25). Although the mechanism of this long-lasting protective phenomenon is not precisely understood, it has been shown that a key role is played by mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}) (3, 31, 48) and protein kinase C (PKC) (13, 28). Emerging evidence based on studies of preconditioning suggests that the link between PKC activation and mitoK_{ATP} opening are reactive oxygen species (ROS) formed during the trigger phase in various forms of protection (6, 26, 35, 47) although their source and the sequence of signaling events is a matter of debate. CIH is also associated with oxidative stress (7, 8), and increased ROS generation may be implicated in the induction of its cardioprotective mechanism. We have shown previously that in rats exposed simultaneously to hypoxia and hypercapnia, which is known to reduce oxidative stress (27), the infarct size-limiting effect of hypoxic adaptation was blunted (30). Increased ROS production also appears to be involved in adverse effects of chronic hypoxia such as the structural remodeling of pulmonary vessels and resulting pulmonary hypertension because hypercapnia or antioxidants attenuated these pathological manifestations (22, 25, 32).

It is well known that ROS-induced acute preconditioning is mediated by activation of PKC (4, 43). Therefore, the present study was designed to verify the hypothesis that oxidative stress acting during the long-term adaptation to hypoxia contributes to the development of increased cardiac ischemic tolerance in a PKC-dependent manner. Effects of chronic treatment with the antioxidant N-acetylcysteine (NAC) on myocardial abundance and sub-cellular distribution of PKC isoforms- δ and - ϵ , and the size of myocardial infarction induced by acute

coronary artery occlusion were compared in rats adapted to CIH and in normoxic controls. Moreover, activities of major antioxidant enzymes in the myocardium were determined.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (250 - 280 g body weight) were exposed to intermittent hypobaric hypoxia corresponding to the altitude of 7000 m for 8 h/day, 5 days a week (Fig. 1). Barometric pressure (P_B) was lowered stepwise, so that the level equivalent to an altitude of 7000 m ($P_B = 308$ mm Hg, 41 kPa; $PO_2 = 65$ mm Hg, 8.6 kPa) was reached after 13 exposures. The total number of exposures was 24-30 to allow for successive processing of animals in physiological experiments; no appreciable changes of hypoxia-induced responses occurred within this interval. A subgroup of the animals received NAC by subcutaneous injections in a dose of 100 mg/kg daily before the hypoxic exposure; the remaining rats received the same volume (2 ml/kg) of saline. The control group of animals was kept for the same period of time at P_B and PO_2 equivalent to an altitude of 200 m ($P_B = 742$ mm Hg, 99 kPa; $PO_2 = 155$ mm Hg, 20.7 kPa); a subgroup was treated with NAC or saline in a corresponding manner. All animals had free access to water and a standard laboratory diet. The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

All animals were employed on the next day following the last hypoxic exposure and/or NAC injection. Hematocrit was measured in the tail blood. The animals assigned to biochemical analyses were sacrificed by de-capitation, their hearts were rapidly excised, washed in cold (0 °C) saline and dissected into the right (RV) and left (LV) free ventricular walls and the septum. All parts were weighed and the left ventricles were frozen in liquid nitrogen and stored at -80 °C until use. All of the chemicals were purchased from Sigma, unless otherwise indicated.

Infarct size determination. Animals were subjected to myocardial ischemia/reperfusion as described previously (29). Anesthetized (sodium pentobarbital, 60 mg/kg i.p., Sanofi) rats were ventilated (Columbus Instruments) with room air at 68 strokes/min (tidal volume of 1.2 ml/100 g body weight). Blood pressure in the left carotid artery was measured (Gould P23Gb) and subsequently analyzed by our custom-designed software. The rectal temperature was maintained between 36.5 and 37.5 °C by a heated table throughout the experiment.

Left thoracotomy was performed and a polyester suture 6/0 (Ethibond - Ethicon) was placed around the left anterior descending coronary artery about 1-2 mm distal to its origin. After 10-min stabilization, regional myocardial ischemia was induced by the tightening of the suture threaded through a polyethylene tube. After a 20-min occlusion period, the ligature was released and reperfusion of previously ischemic tissue continued for 3 h. Then the hearts were excised and washed with saline through the aorta. The infarct area and the area at risk were delineated by perfusion with 2,3,5-triphenyltetrazolium chloride and potassium permanganate (after coronary artery occlusion), respectively. The hearts were cut into slices 1 mm thick and fixed in formaldehyde solution. The size of the infarct area (IA), the size of the area at risk (AR) and the size of the LV were determined by computerized planimetry. The IA was normalized to the AR (IA/AR) and the AR was normalized to the LV (AR/LV).

Tissue fractionation and Western blot analysis of PKC isoforms. Frozen LV myocardium was pulverized to a fine powder at the temperature of liquid nitrogen, followed by Potter-Elvehjem homogenization in 8 volumes of ice-cold buffer composed of (in mmol/l): 12.5 Tris-HCl (pH 7.4), 250 sucrose, 2.5 EGTA, 1 EDTA, 100 NaF, 5 DTT, 0.3 phenylmethylsulfonyl fluoride, 0.2 leupeptin, and 0.02 aprotinin. The homogenate was centrifuged at 100,000 × g for 90 min. The resulting pellet represented the particulate fraction; the supernatant was the

cytosolic fraction. The homogenate and pellet of the particulate fraction were re-suspended in homogenization buffer containing 1 % Triton X-100 held on ice for 90 min and then centrifuged at $100,000 \times g$ for further 90 min. The resulting detergent-treated supernatants were used for immunoblot analyses. Triton X-100 was added to the cytosolic fraction to reach the final concentration of 1 %. Protein content was determined according to Lowry's assay modified by Peterson (37).

Detergent-treated extracts of sub-cellular fractions were subjected to SDS-PAGE electrophoresis on 8 % bis-acrylamide polyacrylamide gel at 20 mA/gel for 90 min on a Mini-Protean II apparatus (Bio-Rad). After electrophoresis, the resolved proteins were transferred to a nitro-cellulose membrane (Amersham Int.). Equal protein transfer efficiency was verified by staining with Ponceau S. After blocking with 5 % dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS), the membranes were immunoblotted using the ECL detection system (Amersham Int.) as previously described (28). Samples from all experimental groups compared were run on the same gel and quantified on the same membrane. To ensure the specificity of PKC- δ and PKC- ϵ immunoreactive proteins, pre-stained MW protein standards (Fluka), recombinant human PKC- δ and PKC- ϵ standards (Sigma), rat brain extract and the respective blocking immunizing peptides (Sigma) were used.

Measurement of antioxidant enzyme activities. Myocardium was pulverized and homogenized as described above. The homogenate was clarified by centrifugation at $5000 \times g$ for 10 min. Catalase (CAT) activity was measured by the method of Aebi (1). The rate of hydrogen peroxide decomposition was monitored spectrophotometrically at 240 nm in 50 mM phosphate buffer (pH 7.0) containing 10 mM hydrogen peroxide at 28 °C.

Glutathione peroxidase (GPX) activity was determined by the indirect procedure described by Paglia and Valentine (34). GSSG was produced by GPX reaction and immediately

reduced by NADPH in the presence of glutathione reductase. The rate of NADPH consumption was recorded at 340 nm as a measure of GSSG formation. The reaction was conducted in 1 M Tris-HCl buffer containing 5 mM Na₂EDTA, 2 mM NADPH, 20mM GSH, 10 U/ml glutathione reductase and started by the addition of t-butyl hydroperoxide. Consumption of NADPH was calculated using mM extinction coefficient for NADPH of 6.22.

Total superoxide dismutase (SOD) activity was determined by the modified nitroblue tetrazolium method (14). Xanthine–xanthine oxidase reaction was utilized to generate a superoxide flux. Nitroblue tetrazolium reduction by superoxide anion to blue formazan was measured spectrophotometrically at 540 nm (28 °C). Chloroform-ethanol extracts of homogenates were then used to determine SOD activity. The assay contained the following reagents: 0.1 mM phosphate buffer (pH 7.8), 4 g/l bovine serum albumin, 2 mg/ml nitroblue tetrazolium, and 1 mM xanthine. Manganese SOD (Mn SOD) activity was quantified in the presence of 5 mM NaCN, the selective inhibitor of copper-zinc SOD (41).

Measurement of glutathione concentration. Myocardium was homogenized in 1 % picric acid using a glass-Teflon device and the homogenate was centrifuged at 10,000 × g for 10 min. Concentration of total glutathione in supernatant was determined spectrophotometrically at 412 nM using glutathione reductase-coupled enzymic assay at 30 °C (16). The assay contained the following reagents: 0.2 mmol/l NADPH, 100 mmol/l phosphate, 5 mmol/l EDTA, 0.6 mmol/l 5,5-dithio-bis(2-nitrobenzoic acid), and 1 kU/l glutathione reductase. Oxidized glutathione (glutathione disulfide, GSSG) was measured by masking the reduced glutathione (GSH) with 2-vinylpyridine. The ratio of GSH/GSSG was taken as a measure of tissue oxidative stress.

Measurement of lipofuscin-like pigments. Additional experiments were performed to verify that CIH is associated with increased oxidative stress and NAC treatment prevents this

effect. A separate group of rats was exposed to hypobaric hypoxia corresponding to the altitude of 5,500 m ($P_B = 379$ mm Hg, 50.5 kPa; $PO_2 = 79$ mm Hg, 10.5 kPa) for 8 h/day during 3 consecutive days. Hypoxic and normoxic animals were treated with NAC or saline as described above and employed the next day following the 3rd hypoxic exposure. The frozen left ventricular myocardium was lyophilized, pulverized and extracted for 1 h in a chloroform:methanol mixture (1:2, v/v). The samples were centrifuged at $2000 \times g$ for 10 min, the bottom chloroform layer was rinsed twice with water and used for the measurement of the concentration of lipofuscin-like pigments (LFP).

LFP are fluorescent end products of lipid peroxidation (9) that have been widely used as an indicator of tissue oxidative stress. Fluorescence emission and excitation spectra of chloroform extracts were measured on the Perkin-Elmer LS-5 fluorometer as previously described (46). The excitation spectra were measured in the range of 250-500 nm for emission adjusted between 300-500 nm with a step of 10 nm. The fluorometer was calibrated with the standard No. 2 of the instrument manufacturer and the LFP concentration was expressed in relative fluorescence units (rfu) per g of dry tissue weight.

Data analysis. The results are expressed as means \pm SE. Differences in the infarct size between the groups were compared by the Mann-Whitney U test. One-way ANOVA and subsequent Student–Newman-Keuls test were used for comparison of differences in parametric variables between the groups. Differences were assumed as statistically significant when $P < 0.05$.

RESULTS

Weight parameters and hematocrit. In line with our previous studies, adaptation of rats to CIH led to a marked increase in hematocrit values and a significant retardation of body growth

compared with age-matched normoxic animals. Treatment with NAC during the adaptation period slightly decreased the body weight in both normoxic and chronically hypoxic groups and did not affect the level of the hematocrit. The heart weight of chronically hypoxic rats increased due to hypertrophy of both ventricles. The RV weight normalized to body weight increased to 177 % and that of the LV to 128 % of the respective normoxic values. NAC-treatment had no effect on heart weight parameters (Table 1).

Myocardial infarct size. The normalized area at risk (AR/LV) did not significantly differ between the groups (Table 1). CIH decreased the infarct size from 56.7 ± 4.5 % of the AR in the normoxic control group to 27.7 ± 4.9 %. NAC treatment decreased IA/AR in the normoxic animals to 42.0 ± 3.4 % but it abolished protection induced by CIH (to 41.1 ± 4.9 %). The IA/AR did not differ between the NAC-treated groups (Fig. 2).

CIH increased mean arterial blood pressure (MAP) compared with normoxic groups, and the higher level of MAP persisted in the course of ischemia and reperfusion. In normoxic controls, MAP significantly decreased at the end of reperfusion compared with baseline values. This decrease was prevented by both NAC treatment and adaptation to hypoxia. Heart rate did not differ between the groups and it significantly decreased at the end of reperfusion in all groups (Table 2).

Myocardial glutathione, LFP, and antioxidant enzyme activities. Table 3 summarizes myocardial activities of total SOD, Mn SOD, CAT and GPX. Neither CIH nor NAC treatment induced an appreciable effect on these enzymes.

CIH did not change the myocardial concentration of total glutathione but it significantly increased the proportion of GSSG and decreased the GSH/GSSG ratio. NAC treatment prevented

the effect of CIH on GSH/GSSG ratio and it increased the concentration of total glutathione without affecting the ratio in normoxic hearts (Table 3).

Myocardial concentration of LFP significantly increased already after 3 daily exposures to hypoxia of 5,500 m and NAC treatment prevented this effect (Table 3).

Expression and distribution of PKC isoforms. Immunoreactivities of PKC- δ and PKC- ϵ were detected on Western blots as single bands that were confirmed by the respective blocking peptides, recombinant human PKC standards, and a positive control from rat brain homogenate (Fig. 3).

CIH significantly increased the relative protein content of PKC- δ in the homogenate from the myocardium (Fig. 4A). This effect was predominantly due to increased abundance of the isoform in the particulate fraction (Fig. 4C). NAC treatment did not affect PKC- δ expression and distribution in normoxic hearts but it prevented its up-regulation by CIH in both homogenate and the particulate fraction.

CIH tended to decrease the relative protein content of PKC- ϵ in the homogenate but the difference reached statistical significance only in a comparison of NAC-treated normoxic with hypoxic groups (Fig. 4B). The abundance of the isoform in the particulate fraction of chronically hypoxic hearts was also significantly lower than in normoxic hearts (Fig. 4D). NAC treatment had no appreciable effect on PKC- ϵ expression.

DISCUSSION

In this study, we demonstrated for the first time that the antioxidant NAC completely prevented the development of cardioprotection in chronically hypoxic rats. Beneficial reduction of myocardial infarct size induced by CIH did not appear in animals treated with NAC every day

before the hypoxic exposure during the whole 5-week adaptation period. The decrease in myocardial GSH/GSSG ratio, which reflects tissue oxidative stress in chronically hypoxic animals, was eliminated by NAC treatment. Myocardial concentration of LFP, another marker of oxidative stress, was elevated already after 3 daily hypoxic exposures and this effect was absent in NAC-treated hypoxic animals. It is in agreement with our previous observation that the infarct size-limiting effect of chronic hypoxia is attenuated by increased concentration of CO₂ in the air during the period of adaptation to hypoxia (30); the increased CO₂ level is considered to act by a decrease of oxidative stress as well (27). Besides protection by CIH, a growing body of evidence indicates that ROS are also involved in intracellular signaling cascades of various forms of early and delayed preconditioning (e.g. 2, 6, 10-12, 20, 45). It appears, therefore, that the generation of the ROS signal before ischemia/reperfusion insult plays an important role in the induction of both short-term and long-term protective programs.

In the past, ROS were considered solely injurious but now it is generally accepted that they may exert both deleterious and beneficial actions (5). Our observation of opposite effects of NAC treatment on ischemic tolerance of normoxic and chronically hypoxic hearts, i.e. decreased and increased infarct size, respectively, is in line with this concept. Infarct size was about the same in the two NAC-treated groups, which means that hearts of treated hypoxic rats were still moderately protected when compared to those of untreated normoxic rats. It suggests that the ischemic tolerance of NAC-treated chronically hypoxic hearts resulted from an interplay between the protective action of the antioxidant and the abrogation of the protective adaptive response induced by CIH. Generally, this dual effect might, at least partially, explain why clinical trials with antioxidants failed to confirm promising data obtained in a number of animal studies. It is obvious that beneficial consequences of antioxidant supplementation in normal healthy myocardium cannot be used to predict an outcome in adapted or diseased hearts.

Cardioprotective properties of NAC have been demonstrated in several experimental studies using various *in vivo* or *in vitro* models (15, 23, 40). NAC is a sulfhydryl-containing compound that exerts its complex antioxidant effect both through direct interaction with ROS and as a precursor of L-cysteine and glutathione. In this study, we assessed cardiac ischemic tolerance on the next day following the last administration of NAC. It seems unlikely that direct scavenging activity of NAC itself was responsible for its protective effect at this time as the elimination half-life of total plasma NAC is about 2 h in rats (18). NAC is rapidly converted to L-cysteine, which is also cardioprotective as a ROS scavenger (39) or can enter in the synthesis of glutathione, a central component of the cellular antioxidant defense system. The potential role of glutathione in the infarct size-limiting effect of NAC treatment in normoxic animals cannot be excluded as its total myocardial concentration was increased although the GSH/GSSG ratio remained unchanged in our study. In addition, NAC increases nitric oxide availability by scavenging ROS and stimulating endothelial nitric oxide synthase activity and protein expression in the heart (36) that might contribute to the protective effect of a prolonged treatment.

Adaptation to CIH led to a marked up-regulation of PKC- δ that was significant in both myocardial homogenate and particulate fraction. This observation confirms the results of our recent study which demonstrated that CIH-induced increase in the relative protein content of PKC- δ was most prominent in mitochondrial and nuclear fractions (28). We also showed that the PKC- δ isoform-selective inhibitor rottlerin, administered before the acute ischemia/reperfusion insult, attenuated the infarct size-limiting effect of CIH, suggesting that this isoform is involved in the cardioprotective mechanism. The novel finding of the present study is that the preventive treatment of chronically hypoxic rats with NAC eliminated the up-regulation of PKC- δ . This observation suggests that the induction of this isoform during the adaptation period is dependent on oxidative stress. The absence of both protection and PKC- δ up-regulation in NAC-treated

hypoxic animals further supports our previous conclusion regarding the role of this isoform in increased ischemic tolerance of chronically hypoxic hearts (28).

PKC- δ may be both protective and detrimental as reported by a growing number of studies. It appears that protective effects of PKC- δ are manifested when the enzyme is activated well before ischemia/reperfusion insult (19). This condition was satisfied in our experiments. Consequences of PKC- δ activation also depend on its localization to various sub-cellular compartments that is controlled by phosphorylation at multiple sites (42). For example, phosphorylation of PKC- δ at serine-643 is associated with its translocation to mitochondria and activation of mitoK_{ATP} channels following a protective stimulus (44). Opening of mitoK_{ATP} channels is considered to play a crucial role in various forms of myocardial protection including that afforded by CIH (3, 31, 48). The sequence of signaling events linking ROS, PKC- δ and mitoK_{ATP} channels in the protective mechanism of CIH remains to be elucidated. Nevertheless, our data are compatible with the view that ROS generation precedes PKC- δ activation and mitoK_{ATP} opening as recently demonstrated in cardioprotection induced by the volatile anesthetic sevoflurane (6). Obviously, the infarct size-limiting pathways induced by CIH may also involve other redox-sensitive steps (24) that were not addressed in the present study.

Unlike PKC- δ , the abundance of PKC- ϵ , the key enzyme isoform involved in the mechanism of preconditioning, was rather decreased in the myocardium of chronically hypoxic rats and NAC treatment did not exert any appreciable effect. More detailed analysis performed in our recent study did not reveal any significant change in PKC- ϵ abundance and sub-cellular distribution due to CIH (28). Taken together, these data suggest that this isoform does not seem to play a major role in the increased cardiac ischemic tolerance in our model of severe CIH. In contrast, cardioprotection afforded by permanent chronic hypoxia in neonatal rabbits appears to involve PKC- ϵ activation and translocation (38) suggesting that the role of PKC isoforms differs in species- and/or age-dependent manner. However, we cannot exclude that, apart from PKC- δ ,

other PKC isoform(s) contribute to protection in our experimental model. It should be noted that a moderately increased expression of PKC isoforms- α , - δ and - ϵ was observed in the myocardial particulate fraction isolated from rats adapted to much less severe hypoxia (13).

It has been well documented that ROS can induce myocardial antioxidant enzymes. In particular, the expression and activity of Mn SOD, a key enzyme that converts superoxide to hydrogen peroxide in mitochondria, increase under various conditions associated with oxidative stress. It has been demonstrated to play a role in delayed preconditioning elicited by ischemia, heat stress, inflammatory cytokines or exercise training (e.g. 17, 20, 21); a close correlation exists between the increase in Mn SOD activity and the reduction of infarct size under these conditions (21). Increased activities of Mn SOD and CAT were also observed in hearts of rats exposed to CIH just after birth for 60 days (49). However, the present study failed to detect any effect of long-term adaptation of adult rats to CIH and/or NAC treatment on total myocardial activities of Mn SOD and other major antioxidant enzymes. We cannot exclude that CIH had a stimulatory effect during the first exposures, which disappeared later on when the animals became fully adapted. Nevertheless, the increased ischemic tolerance of adult chronically hypoxic hearts seems unlikely to be mediated by the increased capacity of enzymic antioxidant defense.

In conclusion, oxidative stress acting during adaptation of rats to CIH, play an important role in the induction of endogenous cardioprotective mechanism, which involves the up-regulation of PKC- δ but not PKC- ϵ . Moreover, our data point to a potentially adverse effect of antioxidant supplementation under conditions, which alone evoke ROS-dependent adaptive responses. This might be considered as one of the reasons why clinical data are rather weak and do not justify use of antioxidants for the prevention and treatment of cardiovascular diseases.

GRANTS

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FIGURE LEGENDS

Fig. 1.

Model of chronic intermittent hypoxia and the experimental protocol. Hypoxic animals were subject to hypobaric hypoxia starting at $PO_2 = 119$ mm Hg (equivalent to an altitude of 2,400 m) and decreasing stepwise up to $PO_2 = 65$ mm Hg (equivalent to an altitude of 7000 m) during the first 13 exposures; this level of hypoxia was maintained for additional 11-17 exposures. Full squares indicate daily exposures lasting 8 h. For the remaining period of each day and for 2 days after each 5-day series of hypoxic exposures, the animals were kept at normoxia ($PO_2 = 155$ mm Hg, equivalent to an altitude of 200 m). Vertical lines at the bottom of the graph indicate N-acetylcysteine (NAC) or saline injections given before each hypoxic exposure. Normoxic animals were kept at $PO_2 = 155$ mm Hg during the whole experiment (indicated by a continuous line) and treated with NAC or saline in a corresponding manner. All animals were employed on the next day following the last hypoxic exposure and NAC or saline injection.

Fig. 2.

Myocardial infarct size expressed as a percentage of the area at risk (IA/AR) in control (Cont) and N-acetylcysteine-treated (NAC) rats adapted to chronic hypoxia and in normoxic animals. Values are means \pm SE from 7–10 hearts in each group. * $P < 0.05$ versus corresponding normoxic group, † $P < 0.05$ versus corresponding untreated group.

Fig. 3.

Western blot analysis of PKC isoforms in myocardial homogenate (Hom) and in cytosolic (Cyt) and particulate (Part) fractions. A and B, representative Western blots of PKC- δ and PKC- ϵ , respectively, in fractions from the control normoxic heart in the absence (left part) and presence

(right part) of the respective blocking peptides. C and D, representative Western blots comparing the expression of PKC- δ and PKC- ϵ , respectively, in fractions from normoxic (N), normoxic N-acetylcysteine-treated (N_N), chronically hypoxic (H) and chronically hypoxic N-acetylcysteine-treated (H_N) rats. Total amounts of protein loaded were as follows: PKC- δ - cytosol 16 μ g, particles 5 μ g, homogenate 10 μ g; PKC- ϵ - cytosol 18 μ g, particles 8 μ g, homogenate 14 μ g. For details see *Materials and methods*. Numbers on the right indicate the positions of pre-stained molecular mass standards in kDa. R, recombinant human PKC- δ or PKC- ϵ standards; B, extract from rat brain homogenate.

Fig. 4.

The expression of PKC- δ (A, C) and PKC- ϵ (B, D) in homogenate (A, B), and their distributions between cytosolic and particulate fractions (C, D) from the myocardium of control (Cont) and N-acetylcysteine-treated (NAC) rats adapted to chronic hypoxia and of normoxic animals. Values are expressed as arbitrary units: the sum of densitometric volumes (related to 1 μ g of protein) measured in all groups on the same blot equals 100. Values are means \pm SE from 5 (PKC- δ) or 6 (PKC- ϵ) hearts in each group. * P <0.05 versus corresponding normoxic group, $^\dagger P$ <0.05 versus corresponding untreated group.

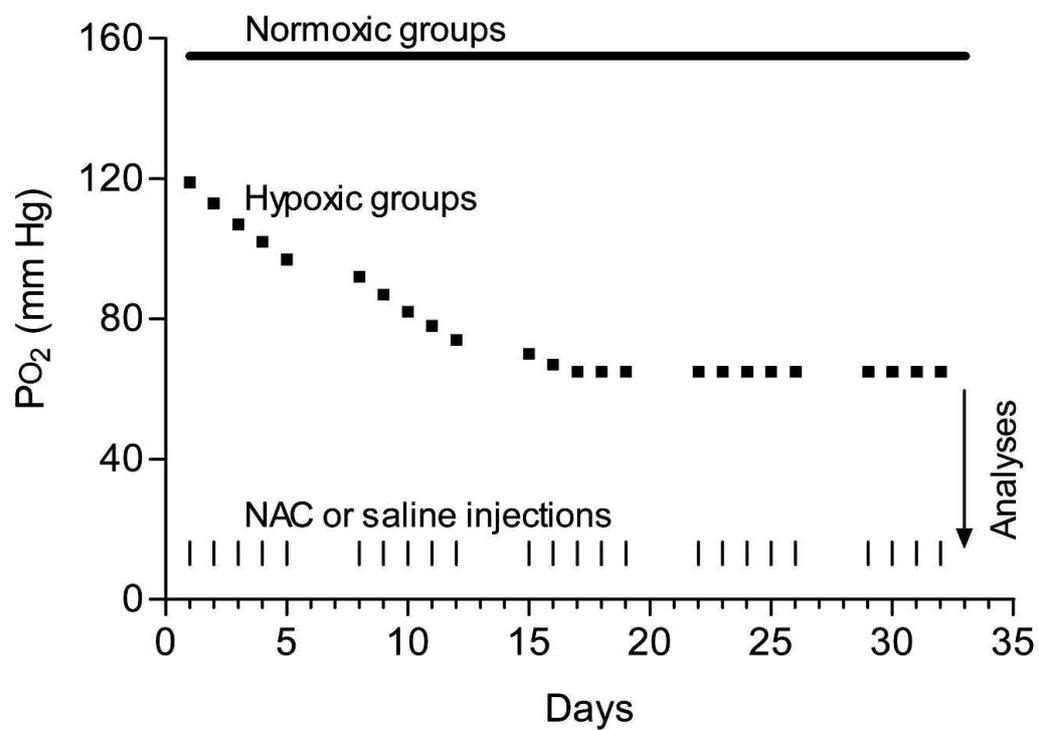


Fig. 1

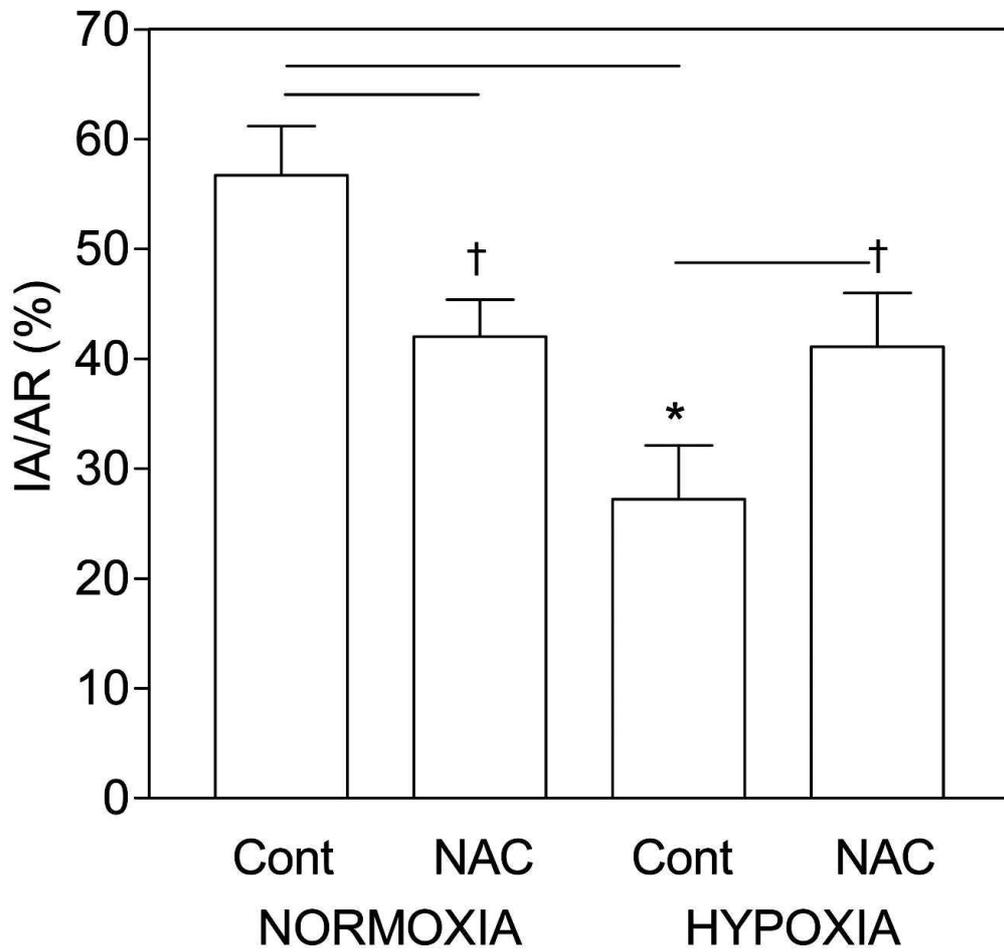


Fig. 2

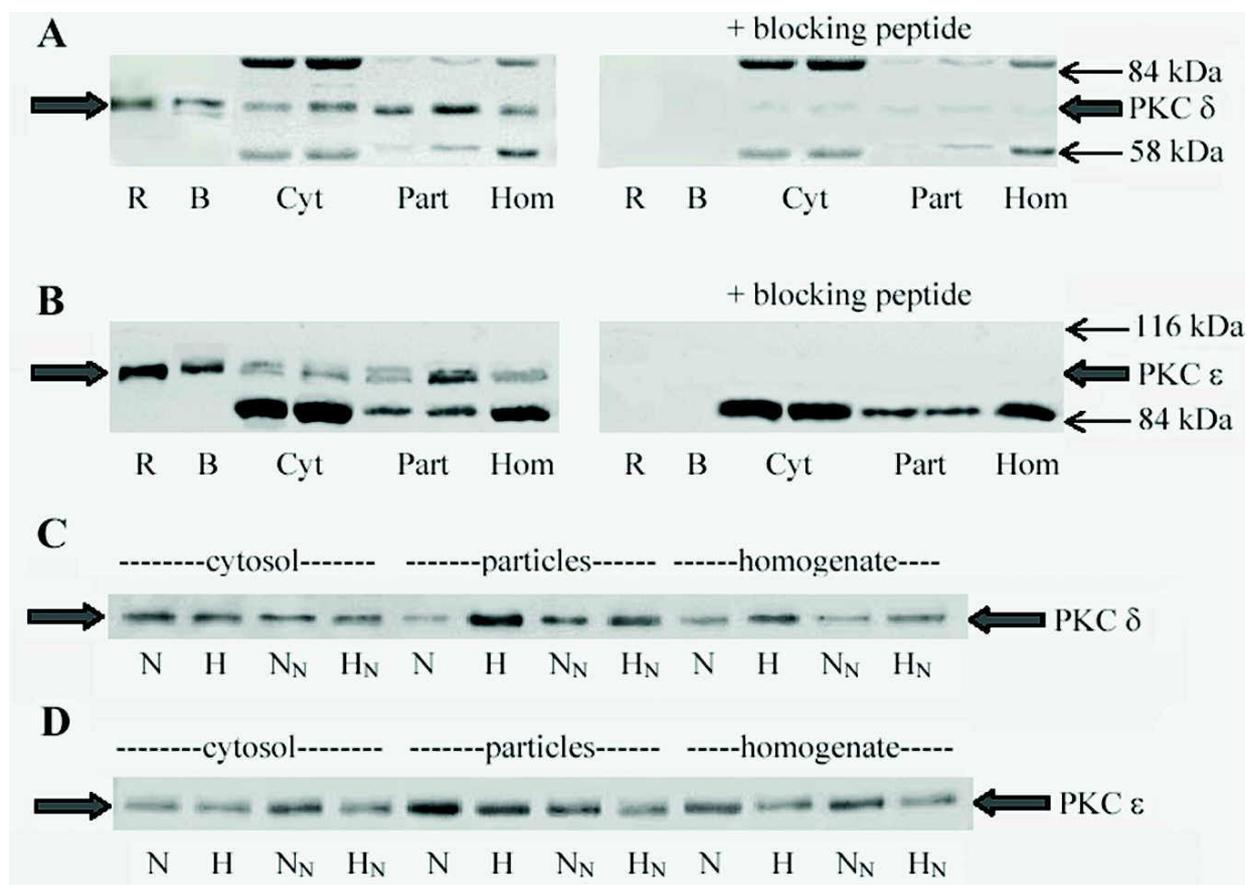


Fig. 3

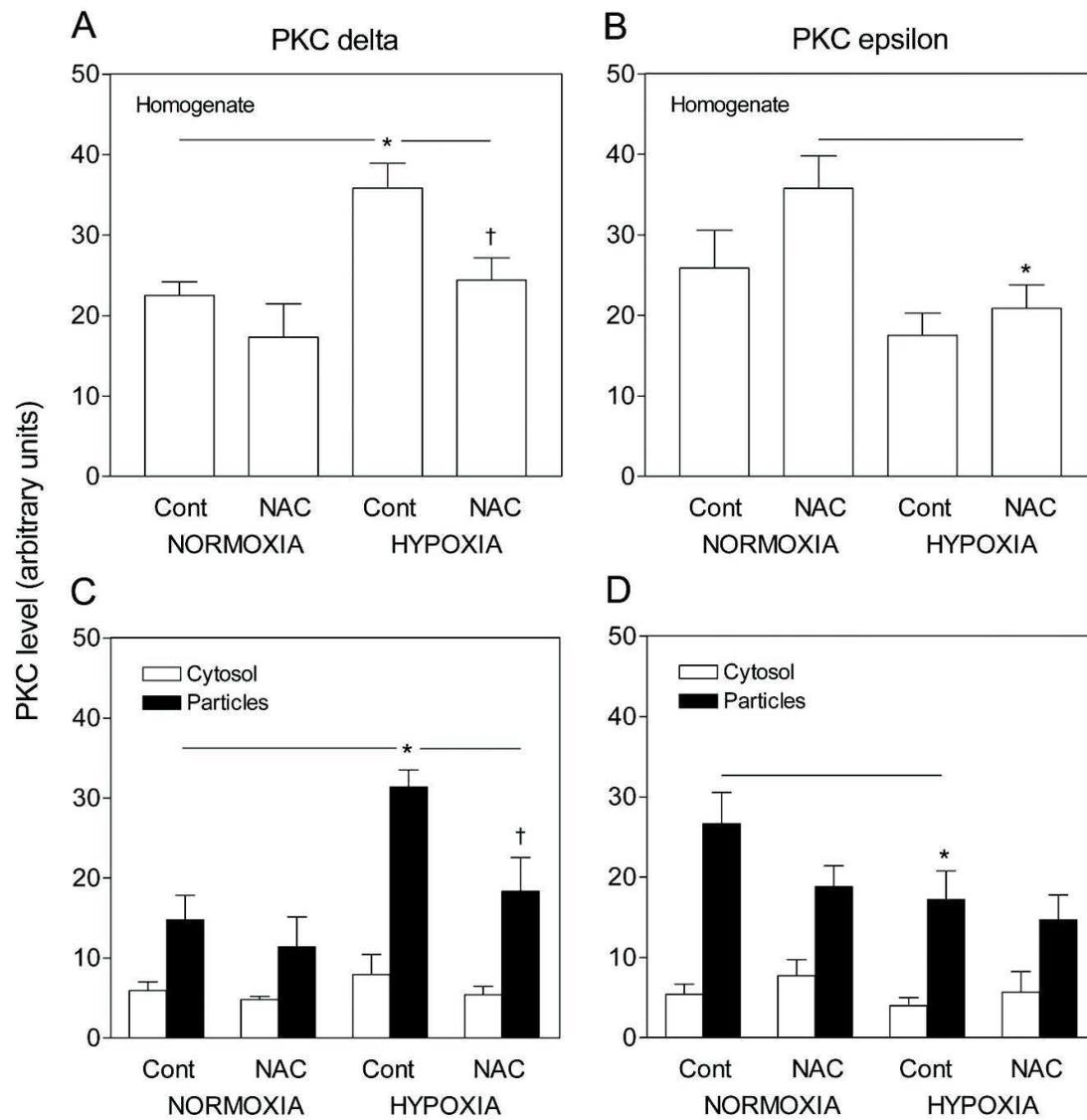


Fig. 4

Table 1. *Body weight and heart weight parameters, hematocrit, and the relative size of the area at risk of infarction.*

Parameter	Normoxia	Normoxia	Hypoxia	Hypoxia
	Controls	NAC	Controls	NAC
BW, g	349 ± 4	331 ± 3 [†]	311 ± 5*	298 ± 4* [†]
RVW, mg	154 ± 4	152 ± 3	244 ± 6*	237 ± 4*
LVW, mg	417 ± 9	396 ± 11	473 ± 17*	465 ± 17*
SW, mg	184 ± 6	179 ± 4	185 ± 5	186 ± 7
RVW/BW, mg/g	0.44 ± 0.01	0.47 ± 0.01	0.78 ± 0.02*	0.80 ± 0.02*
LVW/BW, mg/g	1.19 ± 0.02	1.20 ± 0.03	1.52 ± 0.05*	1.56 ± 0.05*
Haematocrit, %	47.4 ± 0.5	47.0 ± 0.6	70.7 ± 0.7*	71.4 ± 1.1*
AR/LV, %	31.8 ± 4.5	26.5 ± 3.9	34.7 ± 5.7	29.1 ± 3.5

Values are means ± SE from 18-20 rats in each group, except for the parameter AR/LV (area at risk of infarction), which comprise 7-10 hearts in each group. NAC, N-acetylcysteine-treated groups; BW, body weight; RVW, right ventricular weight; LVW, left ventricular weight; SW, septum weight; RVW/BW, relative RV weight; LVW/BW, relative LV weight; * $P < 0.05$ versus corresponding normoxic group; [†] $P < 0.05$ versus corresponding untreated group.

Table 2. *Heart rate and mean arterial blood pressure.*

Group	Pre-ischemic	Ischemia 20 min	Reperfusion 3 h
Heart rate (beats/min)			
Normoxia Controls	418 ± 9	423 ± 8	317 ± 13 [‡]
Normoxia NAC	435 ± 11	428 ± 7	383 ± 10 [‡]
Hypoxia Controls	438 ± 12	432 ± 13	381 ± 16 [‡]
Hypoxia NAC	421 ± 8	419 ± 9	359 ± 12 [‡]
Blood pressure (mm Hg)			
Normoxia Controls	103 ± 6	102 ± 7	86 ± 5 [‡]
Normoxia NAC	102 ± 5	105 ± 6	101 ± 5 [†]
Hypoxia Controls	126 ± 6*	129 ± 6*	127 ± 8*
Hypoxia NAC	131 ± 3*	133 ± 5*	128 ± 4*

Values are means ± SE from 7-10 rats in each group, determined at baseline (pre-ischemic), at the end of 20-min coronary artery occlusion and at the end of the 3-h reperfusion. NAC, N-acetylcysteine-treated groups; * $P < 0.05$ versus corresponding normoxic group; [†] $P < 0.05$ versus corresponding untreated group; [‡] $P < 0.05$ versus baseline.

Table 3. Activities of myocardial antioxidant enzymes, the concentration of total glutathione, the ratio of reduced to oxidized glutathione (GSH/GSSG), and the concentration of lipofuscin-like pigments (LFP).

	Normoxia Controls	Normoxia NAC	Hypoxia Controls	Hypoxia NAC
Total SOD (U/mg protein)	119 ± 8	130 ± 15	141 ± 21	146 ± 23
Mn SOD (U/mg protein)	54.6 ± 5.6	66.4 ± 11.6	57.7 ± 7.1	65.9 ± 10.5
CAT (U/mg protein)	19.2 ± 2.5	17.7 ± 2.0	21.0 ± 2.6	19.8 ± 2.3
GPX (U/mg protein)	1.83 ± 0.22	1.46 ± 0.17	1.78 ± 0.17	1.70 ± 0.20
Total glutathione (μmol/g w.w.)	3.4 ± 0.1	4.3 ± 0.3 [†]	3.3 ± 0.3	3.0 ± 0.1
GSH/GSSG	108 ± 9	98 ± 9	79 ± 6*	121 ± 10 [†]
LFP (rfu/g d.w.)	120 ± 5	121 ± 7	200 ± 22*	135 ± 13 [†]

Values are means ± SE from 7-8 hearts in each group (analyzed after 24 hypoxic exposures), except for the measurement of LFP, which comprise 5 hearts in each group (analyzed after three exposures). NAC, N-acetylcysteine-treated groups; SOD, superoxide dismutase; Mn SOD, manganese SOD; CAT, catalase; GPX, glutathione peroxidase; rfu, relative fluorescence units; * $P < 0.05$ versus corresponding normoxic group; [†] $P < 0.05$ versus corresponding untreated group.