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Research article

Expression and activity patterns of nitric oxide synthases and antioxidant enzymes reveal a substantial heterogeneity between cardiac and vascular aging in the rat

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Abstract

We investigated the effects of aging and ischemia–reperfusion (I/R) injury on the expression and activity of nitric oxide ($^{\bullet}$NO) synthases and superoxide dismutase (SOD) isoforms.To this end we perfused excised hearts from young (6 months old) and old (31–34 months old) rats according to the Langendorff technique. The isolated hearts were, after baseline perfusion for 30 min, either subjected to 20 min of global no-flow ischemia followed by 40 min of reperfusion or were control-perfused (60 min normoxic perfusion).Both MnSOD and Cu,ZnSOD expression remained unchanged with increasing age and remained unaltered by I/R. However, SOD activity decreased from 7.55 ± 0.1 U/mg protein in young hearts to 5.94 ± 0.44 in old hearts ($P < 0.05$). Furthermore, I/R led to a further decrease in enzyme activity (to 6.35 ± 0.41 U/mg protein; $P < 0.05$) in myocardium of young, but not in that of old animals. No changes in myocardial protein-bound 3-nitrotyrosine levels could be detected. Endothelial NOS (eNOS) expression and activity remained unchanged in aged left ventricles, irrespective of I/R injury. This was in steep contrast to peripheral (renal and femoral) arteries obtained from the same animals where a marked age-associated increase of eNOS protein expression could be demonstrated. Inducible NOS expression was undetectable either in the peripheral arteries or in the left ventricle, irrespective of age.In particular when associated with an acute pathology, which is furthermore limited to a certain time frame, changes in the aged myocardium with respect to enzymes crucially involved in maintaining the redox homeostasis, seem to be much less pronounced or even absent compared to the vascular aging process. This may point to heterogeneity in the molecular regulation of the cardiovascular aging process.

Abbreviations: SOD – superoxide dismutase; PGI_2 – prostacyclin; $^{\bullet}$NO – nitric oxide; eNOS – endothelial nitric oxide synthase (NOS III); iNOS – inducible nitric oxide synthase (NOS II); COX – cyclooxygenase; $^{\bullet}O_2^-$ – superoxide; LVDP – left ventricular developed pressure; P – perfusion; I – ischemia; Y – young; O – old; I/R – ischemia/reperfusion; Mn – manganese; Cu,Zn – copper, zinc; cGMP – cyclic guanosine monophosphate; GSH – glutathione

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Introduction

Aging is an important risk factor for the development of cardiovascular disease (Lüscher and Noll 1995). In the vasculature, it is mainly characterized by endothelial dysfunction, which has been demonstrated both in animal studies (Tschudi et al. 1996) and in humans (Zeiher et al. 1993). The underlying mechanisms are not yet completely elucidated, but, based upon the oxidative-stress hypothesis of vascular aging (Stadtman 1992; Finkel and Holbrook 2000), alterations of nitric oxide synthases (NOS) and superoxide ($^{\bullet}O_2^-$)-scavenging antioxidative enzyme systems appear to be involved (van der Loo and Lüscher 2002; Lakatta 2003). Recently, it could be shown that aging led to changes in the phenotype of coronary arterioles including decreased expression of eNOS and cyclooxygenase (COX)-1 with no age-differences in the expression of superoxide dismutases (MnSOD and Cu, ZnSOD) and COX-2 (Csiszar et al. 2003). However, data on the regulation of the L-arginine-cGMP pathway in aged myocardium are controversial as an increased expression and activity of eNOS in whole-heart extracts have also been described (Zieman et al. 2001). Furthermore, aged vessels exhibited increased 3-nitrotyrosine contents (Csiszar et al. 2003) which is a biomarker for the *in vivo* generation of peroxynitrite (Beckman et al. 1992; Zou et al. 1999). Age-associated increase in $^{\bullet}O_2^-$ is thought to be mainly responsible for trapping vasorelaxant $^{\bullet}NO$, a reaction which is currently accepted as the main biological source for peroxynitrite (Goldstein et al. 2000). Peroxynitrite, in turn, is responsible for tyrosine nitration (Reiter et al. 2000) and subsequent inactivation of important enzymes involved in the maintenance of vascular function (Mac Millan-Crow et al. 1996; Zou et al. 1997). Interestingly, several studies have suggested that the regulatory mechanisms involved in the aging process may differ from one vessel type to the other due to vascular bed heterogeneity (Barton et al. 1997; Matz et al. 2000). However, heterogeneity of vascular aging on the one hand and cardiac aging on the other hand has not yet been demonstrated to date. Furthermore, it has not been elucidated if those regulatory mechanisms may change in the aged heart under ischemic conditions, a fact which might possibly explain the well known higher infarct mortality among older patients.

In young rats, it has previously been shown that both $^{\bullet}NO$ and $^{\bullet}O_2^-$ are elevated in response to reperfusion injury (Liu et al. 1997). Subsequent formation of peroxynitrite was increased as indicated by the presence of nitrotyrosine (Liu et al. 1997). Furthermore, prolonged experimental ischemia in young rat hearts leading to myocardial infarction was shown to be associated with an increase of MnSOD mRNA, but not of Cu,ZnSOD, suggesting that the MnSOD gene may be activated as a counterbalancing response (Assem et al. 1997).

This heterogeneous background tempted us to investigate the role of the $^{\bullet}NO$-producing enzyme system as well as of the $^{\bullet}O_2^-$ scavenging enzymes in the particular context of myocardial ischemia–reperfusion injury (stunning) with respect to cardiac aging, which, to our knowledge, has never been investigated before.

Methods

Animals

F1 (F344 × BN) healthy male rats, fed *ad libitum*, were obtained from the National Institutes of Health, National Institute on Aging, Bethesda, MD, USA. Experiments were performed in young (6 months old) and old (31–34 months old) animals. For some experiments, middle-aged animals (18–20 months old) were also used. On the day of the experiment, rats were anesthetized with intraperitoneal injections of ketamine (1 ml/kg body weight) and xylazine (0.5 ml/kg body weight). After medial sternotomy, the hearts were excised, placed on ice-cold Krebs–Ringer bicarbonate solution, and rapidly mounted on the aortic cannula of a Langendorff perfusion chamber. The aorta was excised, placed in cold (4 °C) Krebs–Ringer bicarbonate solution, pH 7.4, and cleaned of adhering tissue. Tissue was then snap frozen in liquid nitrogen until being further processed.

Perfusion procedure and experimental protocol

To induce myocardial stunning, the Langendorff model of retrogradely perfused rat hearts was performed as described previously (Klainguti et al. 2000). Hearts of young and old animals were perfused in a Langendorff perfusion apparatus for 30 min (baseline), then followed in each age class by either

(1) 20 min of total ischemia and 40 min of reperfusion ($n=3$ for hearts from young and from old animals), or
(2) another 60 min of normoxic perfusion ($n=3$ for hearts from young and from old animals).

After completion of the perfusion protocol, cardiac tissue was snap-frozen in liquid nitrogen and stored at −80 °C until analysis. For analysis of expression and activity of antioxidant enzymes and of protein bound 3-nitrotyrosine, tissue of the left ventricle ($n=3$ for each group) was used.

After completion of the perfusion experiments in the Langendorff chamber we investigated potential differences in expression and activity of antioxidant enzymes as well as nitrotyrosine levels in four groups of the left ventricle

(1) young, perfusion only (Y/P; "perfusion");
(2) young ischemia and reperfusion (Y/I; "ischemia");
(3) old, perfusion only (O/P) and
(4) old, ischemia and reperfusion (O/I).

Determination of cardiac eNOS and iNOS protein expression

Frozen cardiac tissue samples of the left ventricle were homogenized and lysed in a Tris–HCl buffer, pH 6.8, containing 2% SDS, 1% urea, 3.6 μM leupeptin and 12.5 mM Tris. After 1 h of incubation on ice and 4 min of sonication in a water bath, the suspension was boiled for 2 min, and the lysate centrifuged at 10,000 *g* at 4 °C for 10 min. Protein concentrations in the lysates were measured using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., UK). Proteins were separated on an SDS 6% polyacrylamide gel at 40 V overnight, and separated proteins were transferred electrophoretically onto polyvinylidine difluoride membranes (Immobilon-P; Millipore) at 200 mA for 40 min. Membranes were blocked with a buffer containing 5% milk powder, 20 mM Tris–HCl (pH 7.50), 150 mM NaCl, and 0.05% Tween 20 (=TBS-T) for 1 h followed by three washes with Tris-buffered saline-Tween (TBS-T). For Western blot analysis, membranes were incubated with the primary monoclonal antibody (rabbit anti-eNOS IgG, Santa Cruz Biotechnology, Inc., or mouse anti-iNOS IgG, Transduction Laboratories) in a dilution of 1:2,000 at room temperature for 2 h. After washing, incubation with peroxidase-labeled anti-rabbit IgG (for detection of iNOS peroxidase-labeled anti-mouse IgG) in a dilution of 1:4,000 followed at room temperature for 90 min. A sample of human umbilical vein endothelial cells was run in parallel as a positive control. Prestained markers were used for molecular mass determinations. Protein expression was detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

NOS activity assay

Frozen cardiac tissue samples were homogenized overnight on ice in 400 μl of a homogenization buffer containing 20 mM HEPES, 200 mM sucrose, 1 mM dithiothreitol, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 2 μg/ml aprotinin. PMSF (0.1 mM final concentration) and [(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (=CHAPS, 20 mM final concentration) were added before sonication. Samples were measured in triplicates. After centrifugation at 100,000*g* for 1 h, supernatants were depleted of endogenous arginine by passage over activated resin. NOS activity was measured by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as described previously (Knowles and Salter 1994). Results are expressed as pmol per μg protein per min. The citrulline assay was carried out in 100 μl of assay buffer (containing: 1.2 mmol/l L-citrulline, 2×10^{-2} mmol/l L-arginine, 0.12 mM NADPH, 10^{-2} mM tetrahydrobiopterin, 1.2 mM $MgCl_2$, 0.24 mM $CaCl_2$, 40 U/ml calmodulin, 10^{-3} mM FAD, 10^{-3} mM FMN, L-[^{14}C]arginine (1.2×10^{-4} mM; 18.5 kBq/ml)) and 18 μl of cytosol from the homogenized samples. Incubations were performed for each sample in the presence or absence of 1 mM

EGTA and/or 1 mM L-NAME. Endothelial cells were used as a positive control. The reaction was eventually terminated by removal of substrate and addition of 1 ml H_2O/Dowex 50×8–400 cationic resin, pH 7.20, and 5 ml of water. After centrifugation of the incubation mix for 3 min at 1,500 rpm, 4 ml of the supernatant in 10 ml of scintillant was examined for [^{14}C]citrulline formation using a scintillation counter.

Determination of cardiac MnSOD and Cu, ZnSOD protein expression

For MnSOD (*Sod 2*) and Cu,ZnSOD (*Sod 1*) determination, homogenisation and extraction were performed as described above. Western blot analysis of MnSOD and Cu,ZnSOD were also performed as described above for NOS. Membranes were incubated with the primary antibody (polyclonal rabbit anti-MnSOD IgG, StressGen Biotechnologies, dilution 1:2,000; or polyclonal rabbit anti-Cu,ZnSOD, Upstate Biotechnology, dilution 1:2,000) at room temperature for 90 min. After three washes in TBS-T, MnSOD and Cu,ZnSOD membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit antibody) (Amersham Pharmacia Biotech) (dilution 1:4,000) for an additional 90 min at room temperature. Finally, membranes were washed three times in TBS-T, and proteins were detected using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

SOD activity assay

Superoxide dismutase was assayed by inhibition of cytochrome *c* reduction according to a previously described method (McCord and Fridovich 1969). The reaction contained 50 μM xanthine, 100 μM EDTA, 10 μM ferri cytochrome *c*, and 50 mM K^+ phosphate, pH 7.80. The measurements were performed in a Jasco V-550 spectrophotometer with a 6-cuvette holder at 25 °C, and absorbance was read at 550 nm. Cytochrome *c* (Sigma Chemicals) was tested for endogenous Cu,Zn SOD contaminations. Xanthine oxidase (Calbiochem) suspensions were centrifuged in an Eppendorf centrifuge for 2 min at maximum speed, and the pellet was dissolved in K^+-phosphate buffer containing protease inhibitor cocktail (Complete, Roche). The activity of the enzyme was adjusted to ~0.025 $\Delta A_{550\ nm}$/min cytochrome *c* reduction. Sample SOD content was assayed by half-maximal inhibition of cytochrome *c* determined in a sample dilution series.

Determination of protein-bound 3-nitrotyrosine levels in cardiac tissue

Tissue extraction and Western blot analysis were performed as described above. To detect nitrated proteins in cardiac tissue, membranes were incubated with the primary antibody (rabbit anti-nitrotyrosine IgG, Upstate Biotechnology, dilution 1:1,000) at room temperature for 2 h. After washing, the blot was incubated with the secondary peroxidase-labeled anti-rabbit antibody (Amersham Pharmacia Biotech) in a dilution of 1:4,000 at room temperature for an additional 90 min. Proteins were detected as described above.

Determination of eNOS and iNOS protein expression in the peripheral vasculature

Frozen aortic segments from young, middle-aged an old animals were pulverized and homogenized in a buffer (pH 7.40) containing 250 mM Tris–HCl. After centrifugation at 10,000*g* and 4 °C for 10 min, the supernatant was transferred to fresh microcentrifuge tubes. Homogenates of animals ($n = 6$) belonging to one age group were pooled according to protein content. To ensure that equal amounts of proteins were loaded in the gels, silver staining was performed using the Silver Staining kit from Amersham Pharmacia Biotech. Western blot analysis for eNOS and iNOS was performed as described for cardiac tissue.

GSH recycling assay

The assay was performed according to the method established by Baker et al. (1990). Briefly, aortic tissue segments were frozen in liquid nitrogen after explantation and homogenized mechanically by using a Mikro-Dismembrator S, Braun Biotech International. The frozen powder was dissolved in PBS and sonicated for 30 s. After centrifugation

at 10,000 *g* for 30 s, total protein contents were adjusted to equal amounts by using the BCA assay. About 200 μl of supernatant were treated with 0.2 vol of 5% SSA (sulfosalicylic acid) for 30 min on ice. After centrifugation at 10,000*g* for 3 min, the supernatant was diluted 5-fold, and each sample was adjusted to pH 7.50.

The buffer reactions were performed in 100 mM sodium phosphate and 1 mM EDTA buffer adjusted to pH 7.50. All reagents for the reaction mixture were freshly prepared before assaying GSH. The mixture consisted of 2.8 ml of 1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 3.75 ml of 1 mM NADPH, 5.85 ml of buffer and 20 U of GSH reductase (Sigma). The GSH-standard curve ranged from 2 μg/ml to 0.02 μg/ml. All samples and standards were kept on ice until loaded onto the microtiter plate. About 50 μl of samples and standards were pipetted into a 96-well microtiter plate, followed by the rapid addition of 100 μl reaction mixture. The plate was read at 405 nm for 5 min with 1 min intervals.

Quantification of blots

Blots were densitometrically quantified using the public domain NIH image 1.60 program developed at the National Institutes of Health (available at: http://rsb.info.nih.gov/nih-image/). All experiments were performed three times, and representative blots are shown.

Statistical analysis

For comparison between two values, statistical analysis was done using Student's *t* test. For multiple comparisons, results were analysed by ANOVA followed by Bonferroni's and Dunn's correction. When applicable, data are presented as means ± SEM. Means were considered significantly different at $P < 0.05$.

Results

Hemodynamics

Hearts were perfused in a non-recirculatory mode at a flow rate of 12 ml/min with Krebs–Henseleit solution. Perfused hearts had a left ventricular developed pressure (LVDP) of approximately 90 mmHg, ischemic and reperfused hearts exhibited a decrease of LVDP, which was, at the end of the protocol, at around 50–60% of the baseline LVDP.

Pattern of expression and activity of nitric oxide synthases

eNOS protein expression in the hearts was neither a function of age nor of ischemia/reperfusion as we found comparable expression levels in all four groups (Figure 1). Furthermore, iNOS expression was undetectable in the hearts of both young and old animals irrespective of exposure to ischemia–reperfusion (data not shown).

We then determined eNOS activity by the conversion of L-[^{14}C]arginine into L-[^{14}C]citrulline. In line with the results on expression levels, no significant change both in total NOS and in eNOS activity between young and old hearts could be observed. Furthermore, although there was a tendency toward higher eNOS activity in postischemic hearts of both young and old animals, no significant difference between postischemic and control-perfused hearts was detectable (eNOS activity: Y/P 265 ± 113, Y/I 755 ± 386, O/P 261 ± 161, O/I 639 ± 283 pmol/min/mg protein; Y/P vs. O/P: $P = 0.98$, Y/I vs. O/I: $P = 0.82$, O/P vs. O/I: $P = 0.31$, Y/P vs. Y/I: $P = 0.19$; data not shown). The lack of any difference between young and old hearts was all the more surprising as we had found in previous work in rat aortas a steep age-dependent increase in eNOS expression and activity (van der Loo et al. 2000).

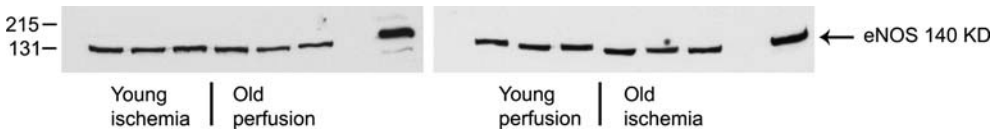

Figure 1. Western blot analysis of eNOS protein expression in cardiac tissue of young and old rats. Before, hearts had either been retrogradely perfused for 60 min (= "perfusion") or, after a baseline perfusion of 30 min, a total global no-flow ischemia of 20 min was eventually followed by 40 min of reperfusion (= "ischemia").

Expression of MnSOD and Cu, ZnSOD

Immunoblot analysis of homogenates from cardiac tissue of old and young animals with polyclonal anti-MnSOD (Figure 2) and anti-Cu, ZnSOD antibody (Figure 3) did not reveal any significant change in enzyme expression either in postischemic or in control-perfused hearts.

Changes in activity of SOD as a function of both age and ischemia–reperfusion

Although expression of MnSOD and Cu,ZnSOD remained unchanged with increasing age, we found a significant decrease in SOD enzyme activity as a function of age. SOD activity decreased from 7.55 ± 0.10 U/mg protein in young rat hearts to 5.97 ± 0.44 U/mg protein in old hearts ($P < 0.05$) (Figure 4). Furthermore, 20 min of total global no-flow ischemia followed by 40 min of normoxic reperfusion also led to a significant decrease in enzyme activity in young hearts (Y/P = 7.55 ± 0.10 U/mg protein, Y/I = 6.35 ± 0.41 U/mg protein; $P < 0.05$). A vice-versa tendency, although not significant, could be observed in old hearts (O/P = 5.97 ± 0.44 U/mg protein, O/I = 6.96 ± 0.18 U/mg protein; $P = 0.08$).

Detection of cardiac 3-nitrotyrosine levels with Western blotting and glutathione levels in cardiac tissue

Neither aging nor ischemia were associated with an increased formation of nitrated tyrosine residues of proteins in cardiac tissue (Figure 5).

Glutathione (GSH) is a highly efficient cellular scavenger of peroxynitrite (Radi et al. 1991) and can prevent Tyr-nitration (Radi 2004). However, in parallel to our results on Tyr-nitration no significant changes of GSH could be found in cardiac tissue, although there was a slight decrease of GSH in the young ischemic–reperfused hearts (Y/P = 0.250 ± 0.052 μg/ml, Y/I = 0.098 ± 0.026 μg/ml, O/P = 0.224 ± 0.049 μg/ml and O/I: 0.314 ± 0.081 μg/ml; Y/P vs. Y/I, Y/P vs. O/P, Y/I vs. O/I and O/P vs. O/I: all ns; data not shown).

Expression pattern of eNOS in the vasculature

The finding of unchanged expression levels of cardiac eNOS with increased age, irrespective of ischemia, was unexpected since we had previously found a striking age-dependent increase of eNOS expression in the aorta using the same animal model of cardiovascular aging (van der Loo et al. 2000). This prompted us to investigate the hypothesis if we could find a further hint for a possible heterogeneity of cardiovascular aging.

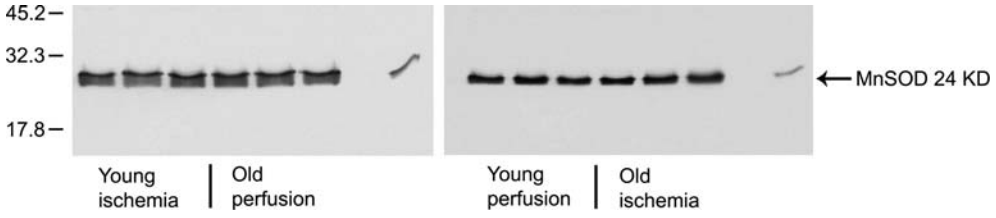

Figure 2. Western blot analysis of MnSOD in homogenates of cardiac tissue of young and old rats after ischemia or after control-perfusion. Molecular weight markers are indicated (in kD) on the left. Recombinant MnSOD served as a positive control.

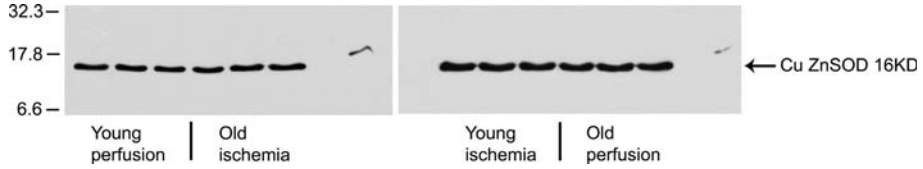

Figure 3. Western blot analysis of Cu,ZnSOD in young and old control-perfused or ischemic-reperfused rat hearts.

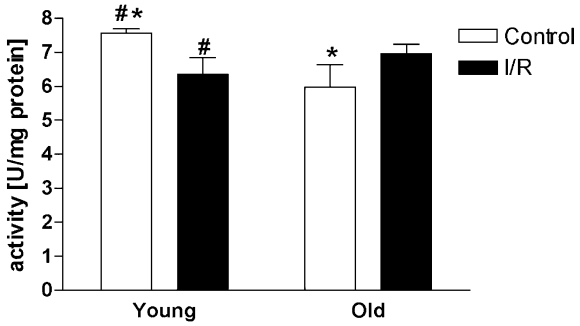

Figure 4. SOD activity in young and old rat hearts that were either ischemic-reperfused or control-perfused. SOD activity was measured by determination of cytochrome *c* reduction as described under Materials and Methods. Significance: Y/P vs. O/P: $^{*}P < 0.05$,; Y/I vs. O/I: ns; Y/P vs. O/I: ns; Y/I vs. O/P: ns; Y/P vs. Y/I $^{\#}P < 0.05$; O/P vs. O/I: ns.

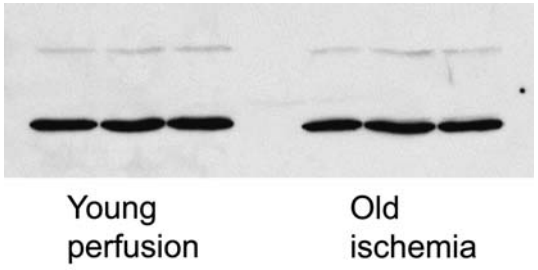

Figure 5. Western blot analysis of protein 3-nitrotyrosine content in young control-perfused hearts and old ischemic-reperfused hearts.

Indeed, when determining eNOS protein expression in other vessels of the peripheral vasculature, we found, in agreement with previous data on the aorta, that eNOS protein expression markedly increased in an age-dependent fashion (Figure 6). Both in the femoral and in the renal artery, eNOS protein expression was two- to threefold increased in old rats as compared to young and middle-aged animals (femoral artery: young vs. old, middle-aged vs. old, and young vs. middle-aged: $P < 0.0001$; renal artery: young vs. old and young vs. middle-aged: $P < 0.0001$, middle-aged vs. old: $P < 0.01$). As in the hearts, no iNOS expression was detectable in the femoral and renal artery irrespective of age.

Discussion

In the present study we investigated putative adaptive mechanisms that may occur especially in the aged heart in association with an acute pathology (e.g. myocardial ischemia and reperfusion injury). To our knowledge, in this context, the important aspect of aging, which, by itself, is one of the most important risk factors for ischemic heart disease (Lakatta 2001), has never been investigated on a molecular basis before.

The rat is by far the most common mammalian model to study cardiovascular aging as, in particular, rats do not develop atherosclerosis which often complicates the analysis of human cardiovascular aging (Folkow and Svanborg 1993). The biological age of young adult and old animals we used corresponds to that of 20-year-old humans and octogenerians, respectively (Stadtman 1992).

Cardiac oxidative stress, as assessed by measuring cardiac malondialdehyde, has previously been found to increase as a function of age (van der Loo et al. 2003). Production of superoxide was also enhanced in ischemic areas of rat hearts after experimental induction of myocardial I/R injury (Liu et al. 1997). Probucol, a potent antioxidant (Kuzuya and Kuzuya 1993), markedly improved post-myocardial infarction survival, an effect which may primarily be attributed to

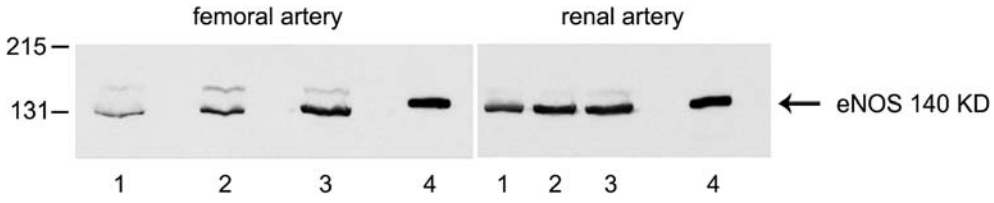

Figure 6. eNOS expression in rat femoral and renal artery. Homogenates from young (lane 1), middle-aged (lane 2), and old rats (lane 3) were separated by SDS-PAGE and analysed by Western blotting for eNOS expression. Human vein endothelial cells served as a positive control (lane 4).

the reduction of oxidative stress and of pro-inflammatory cytokines (Sia et al. 2002). To some extent surprisingly, we could not detect any difference in the cardiac left ventricular expression levels of protective antioxidant enzymes, namely MnSOD and Cu, ZnSOD, irrespective of age and I/R. At first glance, one might argue that myocardial stunning, defined as a reversible dysfunction after a period of ischemia persisting for a certain time despite return of blood flow to normal (Camici and Rimold 1999), is (timely) insufficient to induce any change in protein abundance. This may take longer (at least in an I/R model such as we used it), even though changes in mitochondrial protein abundance have been shown within the first hour after a preconditioning trigger (Lopez and Melov 2002). Changes at the molecular level (e.g. posttranslational changes to proteins ultimately interfering with their function) are believed to, at least in part, contribute to cause the stunning phenomenon. The fact that we observed significant changes in SOD activity favours our concept of a failure of counter-regulatory mechanisms in I/R injury. As expected, SOD activity in aged (control-perfused) hearts was decreased compared with young hearts, a finding which is fully in line with previous data on age-associated increased cardiac oxidative stress. Furthermore, in young myocardium, I/R led to a decrease in SOD activity. Although these changes in activity were significant, their interpretation has to be done with caution, both because of the small sample size and because of the rather small changes observed. The measured changes in activity may partly, but not alone, explain enhanced superoxide production after myocardial I/R injury previously found by others (Liu et al. 1997). Interestingly, in myocardium of old animals, we found a tendency for SOD activity to increase after I/R. This might be an age-inherent, but eventually futile attempt to counter-balance I/R-induced oxidative stress. Surprisingly, when investigating 3-nitrotyrosine levels, a marker for the *in vivo* generation of peroxynitrite, we did not find any difference in protein content irrespective of age and I/R. In parallel the efficient cellular peroxynitrite scavenger glutathione (Radi 2004) remained unchanged. This very interesting finding may be related to another novel and unexpected observation, namely the fact that neither eNOS protein expression nor enzyme activity changed with age or with I/R injury. Therefore, one may assume that $^{\bullet}$NO bioavailability did not change and subsequently, no increased formation of peroxynitrite and hence no peroxynitrite-induced protein modification such as tyrosine nitration took place.

Aging has been found to be associated with an increased prevalence of nitrated tyrosine residues of proteins in intramural coronary arteries and coronary arterioles (Csiszar et al. 2002). These differences may in part arise from the fact that we did our determinations in homogenates of the whole left ventricle (i.e. endocardium, myocardium and epicardium) with a quantitative dominance of myocardium which formed the main part of our analyses and not selectively in coronary arteries and/or arterioles (= coronary microcirculation). The same may be applicable to SOD expression. Furthermore, comparable, but yet different in detail, experimental protocols for I/R may play a role. A lack of change in MnSOD content following I/R in young adult rat hearts has already been described (Subramanian et al. 1993), a finding which is in line with our current data.

In contrast to vascular endothelium, there is only little expression of prostacyclin I_2 (PGI_2) synthase in the myocardium. As PGI_2 synthase is one of the main targets for nitrotyrosine, this may be an important reason why we did not find any change in protein-bound 3-nitrotyrosine levels.

A heterogeneous basal expression pattern of SODs and $^{\bullet}$NO synthases across different regions of the left ventricle and a specific subcellular distribution in the mammalian heart has been described using immunofluorescence and electron microscopy (Brahmajothi and Campbell 1999). eNOS expression was highest in isolated epicardial left ventricular myocytes and was specifically localized to the sarcolemma, suggesting a functional correlation between localization and activity. From our data on overall expression and activity levels we cannot rule out that these specific expression patterns may change with age and/or certain pathological conditions such as I/R injury, thereby entailing potentially important implications for cardiac function.

Apart from our own group (van der Loo et al. 2000), other authors (Goettsch et al. 2001; Cernadas et al. 1998) have also demonstrated an age-related increase of eNOS enzyme expression in the aorta both at the transcriptional and the protein level. The differences to our current findings on aged cardiac tissue are astonishing. Previous data on eNOS expression were obtained in aortic tissue. We now observed changes of a similar magnitude also in the femoral and in the renal artery. Therefore, a heterogeneity of the aging process with respect to the heart (i.e. myocardium) as compared with the vasculature of peripheral arteries may be assumed.

Mitochondrial density in the heart is high, and oxidative stress plays a key role in I/R injury. Mitochondria are key cellular sites both for the production and for the detoxification of reactive oxygen species. Differences in those mitochondrial regulatory systems in the heart as compared to other organs might be involved (Antunes et al. 2002).

The measured alterations described here can obviously not explain the fundamental mechanisms of cardiovascular aging. However, our intriguing findings suggest that mechanisms regulating homeostasis in the aging cardiovascular system become even more complex when the chronic process of aging is exposed to an acute pathology such as I/R injury.

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