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Control of arterial tone after long-term coenzyme Q_{10} supplementation in senescent rats

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1 Age-associated deterioration of arterial function may result from long-lasting oxidative stress. Since coenzyme Q (Q_{10}) has been suggested to protect the vascular endothelium from free radical-induced damage, we investigated the effects of long-term dietary Q_{10} supplementation on arterial function in senescent Wistar rats.

2 At 16 months of age, 18 rats were divided into two groups. The control group was kept on a standard diet while the other group was supplemented with Q_{10} (10 mg kg^{-1} day^{-1}). In addition, nine rats (age 2 months) also ingesting a standard diet were used as the young control group. After 8 study weeks the responses of the mesenteric arterial rings *in vitro* were examined.

3 Endothelium-independent arterial relaxations to isoprenaline and nitroprusside (SNP) were attenuated in aged rats. Increased dietary Q_{10} clearly enhanced the relaxation to isoprenaline, but did not affect the response to SNP. In addition, vasodilation of noradrenaline-precontracted rings to acetylcholine (ACh), which was also impaired in aged vessels, was improved after Q_{10} supplementation. Cyclooxygenase inhibition with diclofenac enhanced the relaxation to ACh only in young rats, while it abolished the difference between the old controls and Q_{10} supplemented rats, suggesting that the improved endothelium-dependent vasodilation observed in Q_{10} supplemented rats was largely mediated by prostacyclin (PGI_2).

4 In conclusion, long-term Q_{10} supplementation improved endothelium-dependent vasodilation and enhanced β-adrenoceptor-mediated arterial relaxation in senescent Wistar rats. The mechanisms underlying the improvement of endothelial function may have included augmented endothelial production of PGI_2, increased sensitivity of smooth muscle to PGI_2, or both.

Keywords: arterial smooth muscle; coenzyme-Q; cyclic adenosine monophosphate; endothelium; prostacyclin; ubiquinone; Wistar rat

Introduction

Aging is associated with a variety of cardiovascular alterations which increase the incidence of pathological processes such as myocardial infarction and stroke (Marin, 1995). The most common age-related change in arterial function is impaired vasodilation, whereas vascular contractile function is usually fairly well maintained (for reviews see Marin, 1995; Folkow & Svanborg, 1993). It is generally agreed that β-adrenoceptor-mediated vasodilation is reduced during aging in humans and animals (Marin, 1995; Folkow & Svanborg, 1993), and a clear decline of endothelium-dependent arterial relaxation has also been found during aging in humans and rats (Taddei *et al*., 1995; Gerhard *et al*., 1996; Delp *et al*., 1995; Atkinson *et al*., 1994). The mechanisms by which aging affects vascular function remain, however, to be established.

Coenzyme Q (ubiquinone) is an endogenous lipid soluble benzoquinone with an established role as an essential component of the mitochondrial electron transport chain and ATP synthesis (Mitchell, 1975). The number of isoprenoid units in the sidechain varies between 1 and 12 in naturally occurring coenzyme Q homologues (Crane, 1977), but the most common homologues in rat are Q_9 and Q_{10}, respectively (Åberg *et al*., 1992). The reduced form of coenzyme Q is an effective antioxidant (Mellors and Tappel, 1966). Both the oxidized and reduced form of coenzyme Q can be found in all cellular membranes where they have been suggested to protect membrane phospholipids and proteins against oxidative damage (Ernster & Dallner, 1995). In concert with this view, a single injection of Q_{10} has recently been reported to protect rat coronary endothelium from free radical-induced damage (Yokoyama *et al*., 1996).

One potential mechanism implicated in the age-related deterioration of vascular function is increased oxidative stress due to a reduction of natural antioxidant defenses (Marin, 1995). Thus, it could be hypothesized that increased dietary coenzyme Q would protect arteries against the age-related changes. To test this hypothesis, we investigated the effects of long-term coenzyme Q supplementation to aged Wistar rats on the *ex vivo* control of arterial tone. Special attention was paid to evaluate the roles of different endothelium-derived mediators in the dilatory responses and to elucidate the possible functional changes in arterial smooth muscle. This study confirmed earlier findings whereby aging was associated with impaired vasodilation, but for the first time showed that both endothelium-dependent and endothelium-independent arterial relaxation could be improved by increased dietary coenzyme Q in aged rats.

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Methods

Animals and experimental design

A total of 27 male Wistar rats were obtained from the experimental animal laboratory of University of Tampere. Before the experiment all rats had been maintained on a standard diet (Altromin 1314, CHR. Petersen A/S, Denmark). The Q_{10} supplemented group (age 16 months, $n=9$) received a daily addition of Q_{10} (10 mg kg^{-1}) for 8 weeks, while the old control group (age 16 months, $n=9$) and the young control group (age 2 months, $n=9$) were kept on a standard diet. Pure coenzyme Q_{10} (Pharma Nord, Vejle, Denmark) was mixed into standard chow by using soybean oil as a vehicle. Soybean oil was also added to the control chow.

Each rat was injected intraperitoneally with heparin (500 IU) 30 min prior to being killed. Rats were anaesthetized by chloralhydrate (250 mg kg^{-1}, intraperitoneally) and decapitated 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 1985). Blood samples were collected from the decapitation line into a 1 ml Eppendorf tube containing 25 μl of heparin. Plasma was immediately separated by cold centrifugation and frozen in liquid nitrogen. Plasma samples were stored at $-70^{\circ}C$ for later coenzyme Q analysis. The superior mesenteric arteries were carefully excised and cleaned of adherent connective tissue.

Coenzyme Q measurements in plasma

The plasma Q_9 and Q_{10} concentrations were determined at an independent laboratory (MILA laboratories, Helsinki, Finland) according to Okamoto *et al.* (1988) with some modifications. The serum samples were extracted with n-propanol (E. Merck, Darmstadt, Germany), and coenzyme Q_7 was added as an internal standard. The coenzymes were reduced with $NaBH_4$ (E. Merck, Darmstadt, Germany) prior to HPLC employing a Gilson 232-401 automated sampler (Gilson Medical Electronics Inc., Villiers le Bel, France). The HPLC equipment consisted of two Wallac 2258 pumps (Pharmacia Biotechnology, Uppsala, Sweden), a Beckman Gold C18-ultraphere column (Beckman Instruments Inc., CA, U.S.A.), a Gilson C18 precolumn, and an ESA electrochemical detector (ESA Inc., MA, U.S.A.).

Mesenteric arterial responses in vitro

The endothelium of the most distal ring was removed by gently rubbing the preparation with a jagged injection needle (Arvola *et al.*, 1992). The rings were placed between stainless steel hooks (diameter 0.3 mm) and suspended in an organ bath chamber (volume 20 ml) in physiological salt solution (PSS, pH 7.4) of the following composition (mM): glucose, 11.1; NaCl 119.0; $NaHCO_3$ 25.0; $CaCl_2$ 1.6; KCl, 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2 and aerated with 95% O_2 and 5% CO_2. The rings were initially equilibrated for 60 min at 37°C with a resting tension of 1.5 g. The force of contraction was measured with an isometric force-displacement transducer and registered on a polygraph (FT03 transducer and model 7E Polygraph; Grass Instrument Co., Quincy, Ma., U.S.A.). The presence of intact endothelium in vascular preparations was confirmed by clear relaxation responses to 1 μM acetylcholine (ACh) in rings precontracted with 1 μM noradrenaline (NA) and the absence of endothelium by the lack of this response. If any relaxation was observed in endothelium-denuded rings, the endothelium was further rubbed.

Receptor and depolarization-mediated contraction

After the equilibration period, the cumulative concentration-response curves for NA and potassium chloride (KCl) were determined. The next concentration of the agonist was added only when the previous level of the response was stable. After the maximal response had been reached, rings were rinsed with PSS and allowed a 20 min recovery period at resting tension.

Endothelium-dependent arterial relaxation after precontraction by NA

Rings were pre-contracted with 1 μM NA, and after the contraction had fully developed, increasing concentrations of ACh were cumulatively added to the organ bath. The next concentration of the agonist was added only when the previous level of the response was stable. After the maximal response had been reached, rings were washed with PSS and equilibrated for 30 min at resting tension with the inhibitor(s). Responses to ACh were then elicited in the presence of 3 μM diclofenac, and in the presence of diclofenac plus 0.1 mM N^G-nitro-L-arginine methyl ester (L-NAME). We have previously evaluated the reproducibility of endothelium-dependent vasodilation in the mesenteric artery of Wistar-Kyoto rat (age 10–12 weeks) by eliciting 3 consecutive cumulative ACh-relaxations in the absence and presence of diclofenac and found these responses to be highly reproducible (pD_2 of the 1st *vs* the 3rd response in the absence of diclofenac: 7.63 ± 0.08 *vs* 7.51 ± 0.07; and maximal relaxation (%): 97.2 ± 0.50 *vs* 97.5 ± 0.64, respectively; pD_2 of the 1st *vs* the 3rd response in the presence of diclofenac: 7.18 ± 0.29 *vs* 7.21 ± 0.03; and maximal relaxation (%): 75.1 ± 4.30 *vs* 79.2 ± 4.98, respectively). In addition, the precontraction induced by 1 μM NA in this arterial preparation is very stable, the change in contractile force during a 20 min contraction being $1.89\pm1.27\%$.

Endothelium-dependent arterial relaxation after precontraction by KCl

Cumulative relaxation responses to ACh were examined after pre-contractions induced by 50 mM KCl. Responses to ACh were then elicited in the presence of 3 μM diclofenac, and in the presence of diclofenac plus 0.1 mM L-NAME. A 30 min incubation was allowed after a new drug was introduced.

Endothelium-independent relaxation and calcium sensitivity during depolarization

After removing the vascular endothelium, the relaxation responses to sodium nitroprusside (SNP) and isoprenaline were examined. The rings were pre-contracted with 1 μM NA, and after the contraction had fully developed, increasing concentrations of the relaxing agents were cumulatively added to the organ bath. After the maximal response had been reached, rings were rinsed with PSS and allowed a 20 min recovery period at resting tension. Thereafter, Ca^{2+} was omitted from the PSS, and the rings were contracted with 10 μM NA to empty the cellular Ca^{2+} stores (Kähönen *et al.*, 1994). When the maximal response had fully developed, the rings were rinsed with Ca^{2+}-free PSS, and once the resting tension was restored the rings were challenged with 125 mM KCl. When the response had reached a plateau, Ca^{2+} was cumulatively added to the organ bath. The procedure was then repeated in the presence of 0.5 nM nifedipine. A 30 min incubation was allowed after nifedipine was introduced.

Table 1 Parameters of contractile responses of isolated mesenteric arterial rings

	US	*Q_{10}S*	*UY*
Maximal contractile force (g)			
Noradrenaline	1.68±0.12*	1.63±0.18*	2.14±0.11
Potassium chloride	1.93±0.10**	2.03±0.13**	2.83±0.19
Inhibitory effect of nifedipine on maximal contraction induced by Ca^{2+} during depolarization (%)	50.4±4.33***	39.1±6.99	26.1±3.02

Values are mean±s.e.mean, $n=9$ for all groups. US, Q_{10}S, UY; unsupplemented senescent, Q_{10}-supplemented senescent, unsupplemented young Wistar rats, respectively.
*$P<0.05$ compared with YC group, **$P<0.01$ compared with YC group, ***$P<0.001$ compared with YC group, Bonferroni test.

Drugs

The following drugs were used: acetylcholine chloride, (±)-isoprenaline hydrochloride, N^G-nitro-L-arginine methyl ester hydrochloride, bitartrate salt of (−)-noradrenaline (Sigma Chemical Co., St. Louis, Mo., U.S.A.), diclofenac (Voltaren injection solution, Ciba-Geigy AG, Basel, Switzerland), nifedipine (Orion Pharmaceutical Co, Espoo, Finland) and sodium nitroprusside (E. Merck AG, Darmstadt, Germany). Stock solutions were made by dissolving the compounds in distilled water, with the exception of nifedipine (in 50% ethanol). All solutions were freshly prepared before use and protected from light. Q_{10} was obtained from Pharma Nord (Vejle, Denmark).

Statistical analysis

Statistical analysis was carried out by a one-way analysis of variance (ANOVA) supported by Bonferroni test in the case of pairwise between-group comparisons (comparisons of Q_9 and Q_{10} concentrations and the values given in Table 1). When the data consisted of repeated observations at successive time points, ANOVA for repeated measurements was applied (comparisons of concentration-response curves within and between panels in Figures 1–4). Unless otherwise indicated the *P* values in the text refer to ANOVA for repeated measurements. Differences were considered significant when $P<0.05$. All results were expressed as mean±s.e.m. The data were analysed with BMDP statistical software (BMDP Inc., Cork, Ireland).

Results

Coenzyme Q concentrations in plasma

The concentration of Q_{10} in plasma was markedly higher ($P<0.0001$, Bonferroni test) in the Q_{10} supplemented group when compared with the old control group (286±25 pmol l^{-1} *vs* 48±30 pmol l^{-1}, respectively), whereas the Q_9 concentrations did not differ between these groups (129±12 *vs* 141±20 pmol l^{-1}).

Mesenteric arterial responses in vitro

In endothelium-denuded rings, the relaxations elicited by SNP, an exogenous NO-donor, as well as those induced by the β-adrenoceptor agonist isoprenaline were attenuated by aging ($P<0.0001$). Interestingly, Q_{10} supplementation clearly improved the relaxation to isoprenaline ($P=0.0001$), but did not affect the response to SNP (Figure 1). In addition, aging was

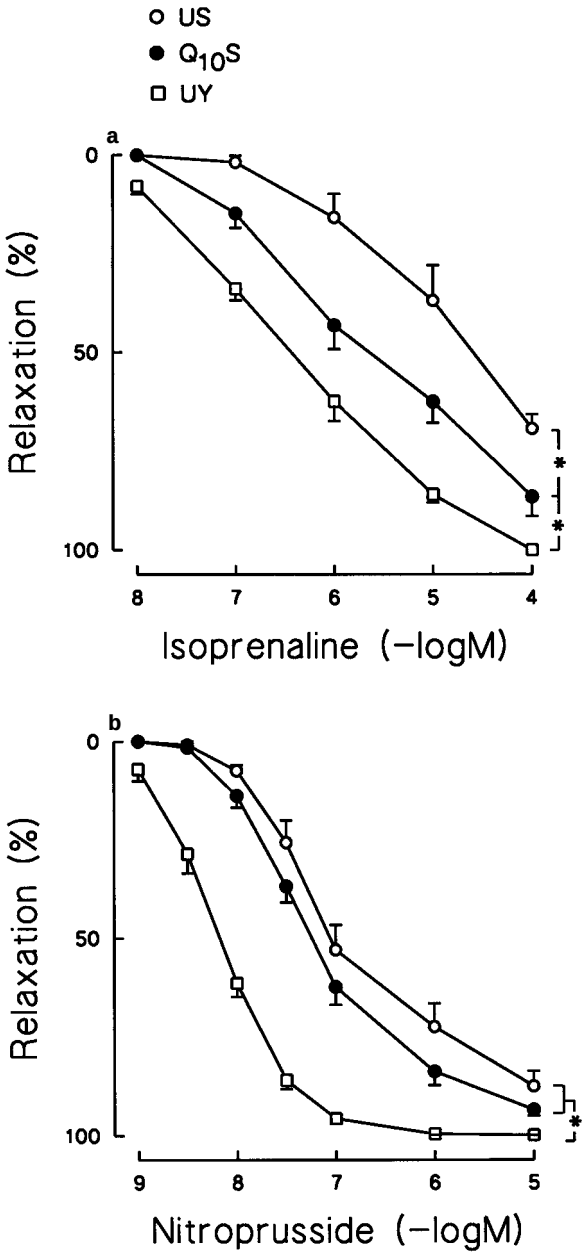

Figure 1 Relaxations to isoprenaline (a) and nitroprusside (b) after precontraction with noradrenaline (1 μM). The responses were elicited in isolated endothelium-denuded mesenteric arterial rings from unsupplemented senescent (US), Q_{10}-supplemented senescent (Q_{10}S) and unsupplemented young (UY) Wistar rats. Symbols indicate means with s.e.±means, $n=9$ in each group; *$P<0.05$, ANOVA for repeated measurements.

associated with impairment of endothelium-mediated vasodilation to ACh in rings precontracted with NA ($P<0.0001$) and this response was also improved by Q_{10} supplementation ($P=0.01$). Cyclooxygenase inhibition with diclofenac enhanced the relaxation to ACh only in young rats ($P<0.0001$, comparison between panel A and B in Figure 2), but abolished the difference between Q_{10} supplemented rats and old control rats. The addition of NO synthase inhibitor L-NAME markedly decreased the relaxation responses to ACh in all groups ($P<0.0001$, comparison between panel B and C in Figure 2), but the relaxation remained more pronounced in young rats than in the old rats.

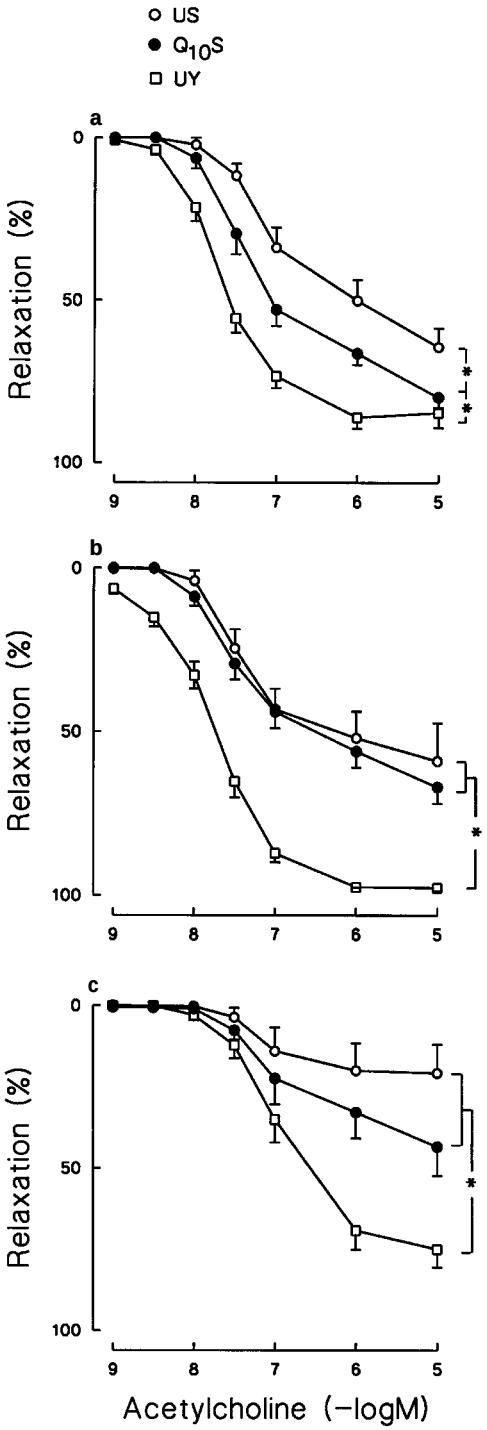

Figure 2 Relaxations to acetylcholine in noradrenaline (1 μM) precontracted isolated endothelium-intact mesenteric arterial rings from unsupplemented senescent (US), Q_{10}-supplemented senescent (Q_{10}S) and unsupplemented young (UY) Wistar rats. The relaxations were induced in the absence (a) and presence (b) of 3 μM diclofenac, and in the presence of diclofenac and 0.1 mM N^G-nitro-L-arginine methyl ester (L-NAME; c). Symbols indicate means with s.e. $\pm$ means, $n=9$ in each group; $*P<0.05$, ANOVA for repeated measurements.

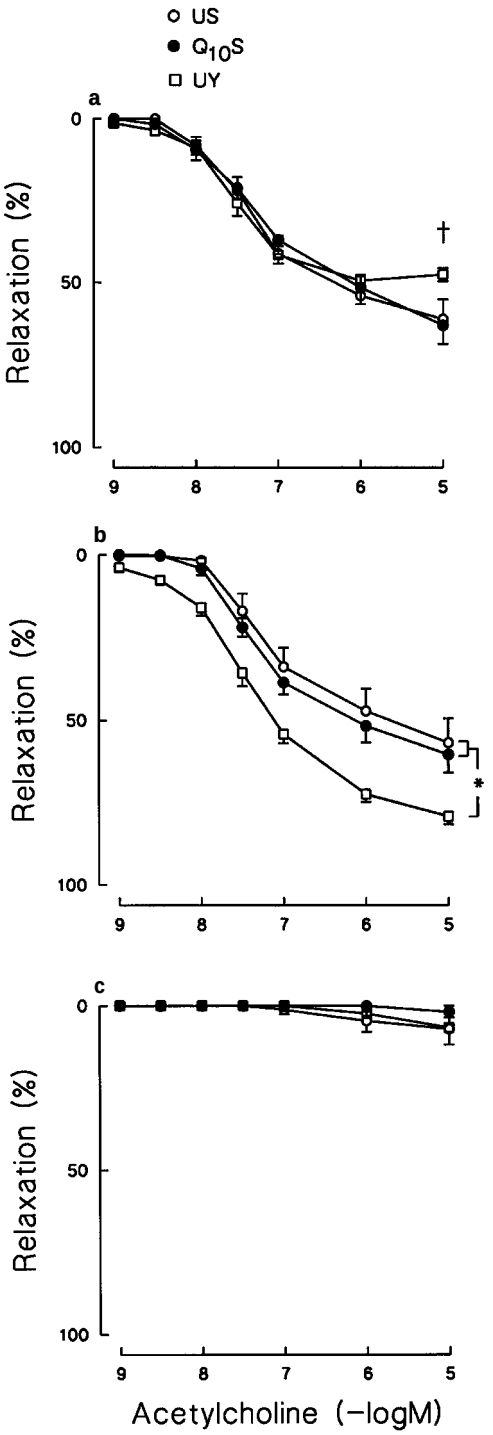

Figure 3 Relaxations to acetylcholine in KCl (50 mM)-precontracted isolated endothelium-intact mesenteric arterial rings from unsupplemented senescent (US), Q_{10}-supplemented senescent (Q_{10}S) and unsupplemented young (UY) Wistar rats. The relaxations were induced in the absence (a) and presence (b) of 3 μM diclofenac, and in the presence of diclofenac and 0.1 mM N^G-nitro-L-arginine methyl ester (L-NAME; c). Symbols indicate means with s.e. $\pm$ means, $n=9$ in each group; $\dagger P<0.05$, Bonferroni test, $*P<0.05$, ANOVA for repeated measurements.

When hyperpolarization of arterial smooth muscle was eliminated by precontractions induced by 50 mM KCl (as described by Adeagbo & Triggle, 1993), no differences were found between the study groups in the relaxation responses to ACh, with the exception of the results with the final concentration (10^{-5} mM ACh). This particular concentration induced a slight contraction in rings obtained from young rats while it further relaxed the rings from the old rats. The addition of diclofenac had no effect on ACh-induced relaxations in the old groups, but again it clearly improved the relaxation in the young group ($P<0.0001$, comparison between panel A and B in Figure 3). The relaxations of KCl-precontracted rings to ACh were completely abolished in all three groups when elicited in the presence of diclofenac and L-NAME (Figure 3).

In endothelium-intact rings, the contractile responses to the receptor-mediated agonist, noradrenaline and to depolarization induced by KCl were comparable in both old groups. Young rats showed higher sensitivity ($P<0.05$) and maximal force ($P<0.05$, Bonferroni test) to both NA and KCl than did the old rats. Sensitivity of smooth muscle to cumulative Ca^{2+} in the absence and presence of nifedipine was also unaltered by Q_{10} supplementation (the response in the presence of nifedipine not shown). Young rats were more sensitive to cumulative Ca^{2+} during depolarization ($P<0.0001$) and more resistant to the inhibitory effect of nifedipine on this response ($P=0.0004$, Bonferroni test; Figure 4, Table 1).

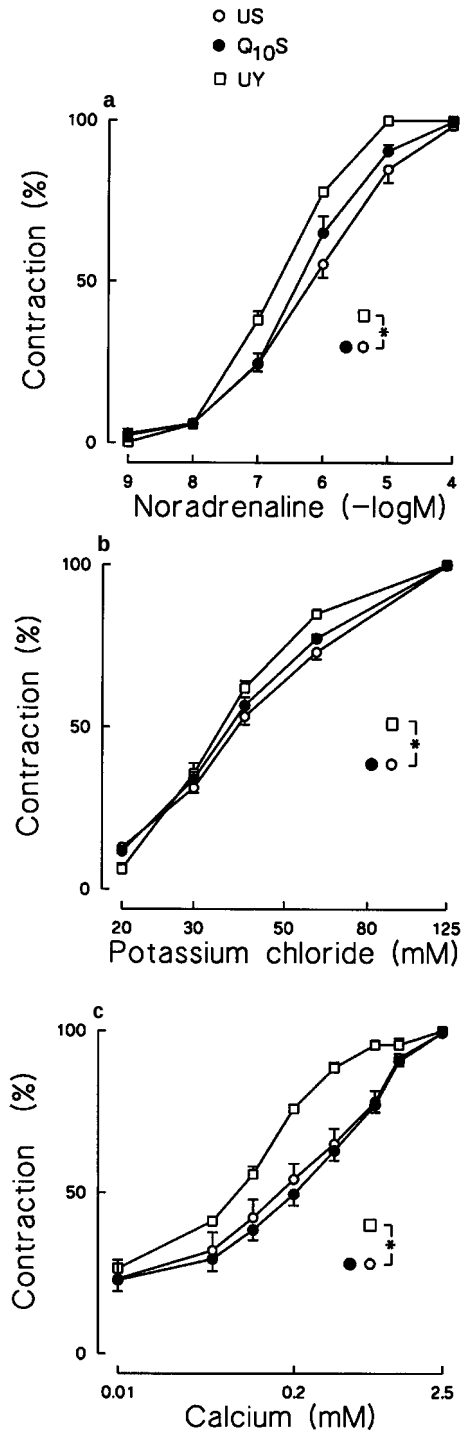

Figure 4 Concentration-response curves of endothelium-intact arterial rings to noradrenaline (a) and KCl (b) from unsupplemented senescent (US), Q_{10}-supplemented senescent (Q_{10}S) and unsupplemented young (UY) Wistar rats. The last panel shows the effect of organ bath calcium concentration on KCl (125 mM)-induced contraction in endothelium-denuded rings (c). Symbols indicate means with s.e. ± means, $n=9$ in each group; $^*P<0.05$, ANOVA for repeated measurements.

Discussion

One of the most common alterations in vascular function associated with aging is impaired β-adrenoceptor-mediated vasodilation (Marin, 1995; Folkow & Svanborg, 1993). In addition, β-adrenoceptor-mediated responsiveness of the heart is also decreased with increasing age (Folkow & Svanborg, 1993). These changes are likely to contribute to the age-related decline of exercise tolerance. In the present study, long-term Q_{10}-supplementation clearly improved the attenuated arterial relaxation to the β-adrenoceptor agonist isoprenaline in senescent Wistar rats.

Stimulation of β-adrenoceptors in vascular smooth muscle leads to the activation of adenylate cyclase, and subsequently to an increase in intracellular cAMP (Bulbring & Tomita, 1987). Aging has been found to be associated with decreased production of cAMP in response to β-adrenoceptor-stimulation (Tsujimoto *et al*., 1986; Marin, 1995; Folkow & Svanborg, 1993). In addition, the relaxation responses to cAMP (induced by forskolin) and dibutyryl cAMP have been found to be lower in the mesenteric arterial rings from old rats than in those from young rats (Tsujimoto *et al*., 1986). These results suggest that the defects underlying the age-related reduction of β-adrenoceptor responsiveness involve both the production of cAMP and the function of cAMP-dependent protein kinases or more distal mechanisms. The cellular mechanisms underlying the observed beneficial effect of coenzyme Q on β-adrenoceptor-mediated vasodilation remain to be studied in the future.

Aging has also been found to attenuate vasodilation induced by the NO-donor nitroprusside in rat mesenteric artery and aorta (Delp *et al*., 1995; Atkinson *et al*., 1994), although contradictory results have also been published (Küng & Lüscher, 1995). In the present study, aging was associated with a decreased relaxation response to nitroprusside. This response was comparable in both of the old groups, suggesting that supplementation with Q_{10} did not affect cGMP-mediated vasodilation. However, it should be kept in mind when interpreting these results that the vasodilatory effect of nitroprusside does not appear to be solely mediated by NO, and therefore, the relaxation induced by nitroprusside cannot be exclusively regarded as NO-mediated (Feelisch, 1991).

ACh is known to relax arterial smooth muscle by releasing several dilatory factors from the vascular endothelium (Furchgot & Vanhoutte, 1989). NO, prostacyclin (PGI_2) and

endothelium-derived hyperpolarizing factor (EDHF) are the major contributors to the ACh-induced vasorelaxation (Busse & Fleming, 1993). Endothelium-mediated vasodilation has been shown to decline with increasing age in humans (Taddei *et al*., 1995; Gerhard *et al*., 1996), as well as in rats (Delp *et al*., 1995; Atkinson *et al*., 1994); this was also confirmed in the present study. Interestingly, we found that the vasodilation to ACh was improved by Q_{10} supplementation in senescent rats. Furthermore, the inhibition of cyclooxygenase (COX) abolished the difference between the two aged groups, suggesting that the enhancement of endothelium-dependent vasodilation after Q_{10} supplementation was largely mediated by PGI_2. The cellular action of PGI_2 are exerted via binding to specific membrane receptors, which like β-receptors, activate adenylate cyclase and subsequently increase the intracellular concentration of cAMP in smooth muscle (see Busse *et al*., 1994). Therefore, the present enhancement of endothelium-mediated vasodilation by Q_{10} can be explained by increased endothelial production of PGI_2 or by the increased sensitivity of arterial smooth muscle to agonists which induce vasorelaxation via an increase in cellular cAMP.

Endothelium-mediated relaxations which remain resistant to both NO synthase (NOS) and COX inhibitions are mediated by EDHF (Cohen & Vanhoutte, 1995). The chemical characteristics of EDHF remain unknown, but functionally this factor is a K^+ channel opener (Cohen & Vanhoutte, 1995), the action of which can be inhibited by K^+ channel blockers or by depolarizing the cell membrane with high concentrations of KCl (Adeagbo & Triggle, 1993). We found that the relaxations of NA-precontracted rings to ACh in the presence of NOS and COX inhibitors were more pronounced in young rats than in old rats. This finding is in agreement with the earlier reports that endothelium-dependent hyperpolarization is decreased by aging in the rat mesenteric artery (Nakashima & Vanhoutte, 1993; Fujii *et al*., 1993).

The differences observed in ACh-induced relaxations during precontractions with NA were no longer detected when the precontractions were elicited by KCl, thereby preventing hyperpolarization. This can be explained by the finding that the response to ACh in young rats is largely dependent upon hyperpolarizing mechanisms, whereas in old rats ACh induces vasodilation mainly via NO (Mantelli *et al*., 1995). Under conditions preventing hyperpolarization, the relaxation induced by ACh did not differ between the old groups, although a clear difference which could be attributed to PGI_2 was observed after precontractions induced by NA. This can be explained by the fact that cAMP-dependent vasorelaxation is also significantly mediated via the activation of K^+ channels and hyperpolarization of smooth muscle (Cohen & Vanhoutte, 1995; Chang, 1997). Furthermore, COX-inhibition enhanced the responses to ACh only in young rats, suggesting that production of COX-derived contracting factors occurs in the endothelium of young rats, whereas the contribution of these factors is minimal in aged rats, as was also suggested earlier by Küng and Lüscher, 1995. The presence of both COX and NOS inhibitors completely abolished the relaxation of KCl-precontracted rings to ACh in all groups, indicating that NO, PGI_2, and EDHF were indeed responsible for the endothelium-mediated relaxations.

Q_{10}-supplementation had no effect on receptor-mediated or depolarization-mediated arterial contractions, nor did it affect the calcium sensitivity of smooth muscle during depolarization or the influence of nifedipine thereupon. Therefore, the improved arterial relaxation following increased dietary Q_{10} supplementation could not be attributed to differences in voltage-dependent Ca^{2+} entry or contractile sensitivity.

In conclusion, long-term supplementation of coenzyme Q_{10} was accompanied by improved endothelium-dependent vasodilation and enhanced β-adrenoceptor-mediated relaxation of arterial smooth muscle in senescent Wistar rats. The mechanisms underlying the improvement of endothelial function may have included augmented endothelial production of PGI_2, increased sensitivity of arterial smooth muscle to PGI_2, or both.

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