FEBS Letters 448 (1999) 206–210 FEBS 21838

Superoxide induces apoptosis in cardiomyocytes, but proliferation and expression of transforming growth factor-β1 in cardiac fibroblasts

Pei-Feng Li, Rainer Dietz, Rüdiger von Harsdorf*

Department of Cardiology, Franz Volhard Clinic, Humboldt University Berlin, and Max-Delbrück-Center for Molecular Medicine, Berlin, Germany

Received 25 February 1999

Abstract Cardiomyocyte apoptosis and cardiac fibroblast proliferation are characteristic features of failing myocardium. Here we investigated the effect of superoxide on the cell fate of cardiomyocytes and cardiac fibroblasts. Cultured rat cardiomyocytes or cardiac fibroblasts were treated with superoxide. In response to superoxide stimulation cardiomyocytes underwent apoptosis as revealed by the increase in histone associated DNA fragmentation and positive to in situ nick end-labeling. In contrast, cardiac fibroblasts were stimulated to proliferate as demonstrated by the increase in DNA synthesis detected by [^{3}H]thymidine incorporation and in cell number. Additionally, Northern blot analysis showed that transforming growth factor-β1, a key factor responsible for myocardial fibrosis, was upregulated in cardiac fibroblasts in response to superoxide stimulation. These data suggest that superoxide can induce such divergent effects as apoptosis in cardiomyocytes and cell growth in cardiac fibroblasts, indicating that it may be a potential factor involved in the pathogenesis of heart failure.

Key words: Cardiomyocyte; Cardiac fibroblast; Superoxide; Apoptosis; Proliferation

1. Introduction

Cardiomyocyte apoptosis has been observed in heart diseases such as dilated cardiomyopathy, ischemic cardiomyopathy, acute myocardial infarction, and cardiac allograft rejection (for review see [1]). Patients afflicted with these diseases have a poor prognosis due to the development of congestive heart failure. Interestingly, there is increasing evidence indicating that oxidative stress is related to heart failure [2,3]. However, it remains to be determined which type of ROS is involved in heart failure. Since there is evidence showing that mice lacking manganese superoxide dismutase die with a dilated cardiomyopathy [4,5], we hypothesized that superoxide (O_2^-) might be a potential candidate.

In the chronic state failing myocardium is characterized by loss of cardiomyocytes and extensive fibrosis. The latter of which may be related to the action of transforming growth factor-β1 (TGF-β1), because TGF-β1 stimulates the synthesis of extracellular matrix components such as collagen, fibronectin, and proteoglycan and inhibits extracellular matrix degradation [6]. It has been suggested that fibroblasts activation and proliferation are responsible for the overproduction of TGF-β1 [7]. Angiotensin II is known to be implicated in the development of myocardial fibrosis and its action in cardiac fibroblasts has been shown to stimulate the autocrine production and release of TGF-β1 [8,9]. To date, only little information exists regarding the identification of stimuli leading to the proliferation of fibroblasts.

Therefore, it was the aim of our study to test whether O_2^- is able to induce both cardiomyocyte death and cardiac fibroblast proliferation and as such may be an important contributor to the development of the characteristic phenotype of failing myocardium. In addition, the gene expression of TGF-β1 was also detected in cardiac fibroblasts in response to superoxide stimulation.

2. Materials and methods

2.1. Materials

Xanthine oxidase, xanthine, hydrogen peroxide (H_2O_2), bovine erythrocyte superoxide dismutase (SOD), bovine liver catalase and anti-α-sarcomeric actin monoclonal antibody were purchased from Sigma (St. Louis, MO, USA). Cell death detection ELISA kit was purchased from Boehringer Mannheim (Germany). Dulbecco's modified Eagle medium F-12 was from Gibco. In situ apoptosis detection kit was purchased from Oncor. Rat TGF-β1 cDNA probe was kindly provided by Dr. Martin Stula (Humboldt-University, Berlin, Germany).

2.2. Cell cultures

Monolayer cultures of neonatal rat cardiac cells were prepared by modifications of the method described by Simpson et al. [10]. Briefly, hearts from 1-day old Wistar rats were dissected, minced, and placed in PBS. The tissue was trypsinized at 37°C in a HEPES-buffered saline solution: 20 mM HEPES-NaOH, pH 7.6, 130 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 4 mM glucose, 0.15% trypsin. Following centrifugation, cells were resuspended in Dulbecco's modified Eagle medium F-12 containing 5% heat-inactivated horse serum, 100 μM ascorbate, 1 μg/ml insulin, 1 μg/ml transferrin, 10 ng/ml selenium, 100 U/ml penicillin, 100 μg/ml streptomycin. The dissociated cells were preplated at 37°C for 1 h and subsequently cultured for 62–72 h in medium containing 0.1 mM bromodeoxyuridine to prevent proliferation of non-myocytes. These cells are hereafter referred to as cardiomyocytes. More than 95% of cells were cardiomyocytes as detected by immunostaining with the α-sarcomeric actin antibody.

In order to obtain cultures of cardiac fibroblasts, cells adherent to the culture dishes were cultured after preplating by adding the normal culture medium except that 10% fetal calf serum was added and bromodeoxyuridine was omitted. Cells used for experiment were at passage level 4–6. In these passages more than 95% of cells were fibroblasts as detected by immunostaining with the ecto-5′11-nucleotidase monoclonal antibody (a generous gift from B. Kaissling [11]). These cells are hereafter referred to as cardiac fibroblasts.

2.3. Exposure of cells to O_2^- generating system or to H_2O_2

Cultured cells were washed twice with Hanks' balanced salt solution (HBSS) at 37°C. Washed cells were incubated at 37°C for 1 h in HBSS containing the indicated concentration of xanthine oxidase

*Corresponding author. Franz-Volhard-Klinik, Universitätsklinikum Charité, Humboldt-Universität, Wiltbergstr. 50, 13125 Berlin, Germany. Fax: (49) (30) 9406 3145.
E-mail: rharsdo@mdc-berlin.de

Abbreviations: SOD, superoxide dismutase; TGF-β1, transforming growth factor-β1; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling; XO/X, xanthine oxidase plus xanthine

PII: S0014-5793(99)00370-1

plus xanthine (XO/X) in the presence of catalase. H_2O_2 treatment was performed under the same conditions. The reaction was stopped by removing the HBSS containing the O_2^- generating system or H_2O_2. The cells were further cultured for the indicated time in freshly prepared culture medium as before treatment. When SOD or catalase were used, they were added simultaneously with XO/X. Control cells were treated under the same conditions without XO/X.

2.4. Analysis of DNA fragmentation

DNA fragmentation was analyzed by cell death detection ELISA. ELISA was performed according to the manufacturer's instructions. Briefly, the anti-histone monoclonal antibody was added to the 96-well ELISA plates and incubated overnight at 4°C. Following recoating, the cytoplasmic fractions were added and incubated for 90 min at room temperature. Bound nucleosomes were detected by the addition of an anti-DNA-peroxidase monoclonal antibody and reacted for 90 min at room temperature. After the addition of the substrate optical density was read with an ELISA reader at 405 nm.

2.5. In situ nick end-labeling and immunofluorescence

The terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assay was used to detect DNA fragmentation in situ. The detection procedures were in accordance with the kit instructions. Briefly, cells were fixed in 4% neutral buffered formalin and subsequently incubated with TDT in the presence of digoxigenin-conjugated dUTP for 1 h at 37°C. After the reaction was terminated the fluorescein-labeled anti-digoxigenin antibody was incubated with samples for 30 min. Following the TUNEL procedure, samples were blocked by 1.4% bovine albumin for 30 min. Subsequently, the anti-α-sarcomeric actin monoclonal antibody and the anti-mouse Ig-rhodamine was incubated on the coverslip for 1 h at 37°C.

2.6. Measurements of DNA synthesis and cell number

Cells were grown in 24-well plates with a seeding density at 2000/cm^2. When cells reached subconfluence they were made quiescent for 48 h in 0.2% serum conditions. After treatment, cells were further cultured for 24 h, and pulse-labeled with [^{3}H]thymidine (1 μCi/ml) for 1 h before the completion of the 24 h incubation period. [^{3}H]Thymidine incorporation into DNA was measured as trichloroacetic acid (TCA)-insoluble radioactivity as described previously with slight modifications [12]. Briefly, cells were washed three times with PBS, and then incubated with 15% TCA at 4°C for 30 min. After aspiration of TCA, cells were washed twice with distilled water. 1 M NaOH was added for 20 min and neutralized with 1 M HCl. The contents of the wells were placed in scintillation vials for counting. For the determination of cell numbers, cells were cultured for 6 days after treatment. The media were changed every 48 h. Cells were suspended with trypsin/EDTA (0.05%/0.5 mM) and counted using a hemocytometer.

2.7. RNA isolation and Northern blot analysis

Total RNA isolation, prehybridization and hybridization were performed as described elsewhere [13]. For chemiluminescent detection, the membrane was blocked for 30 min in 2.5% blocking reagent. The membrane was then incubated for 30 min with an anti-digoxigenin

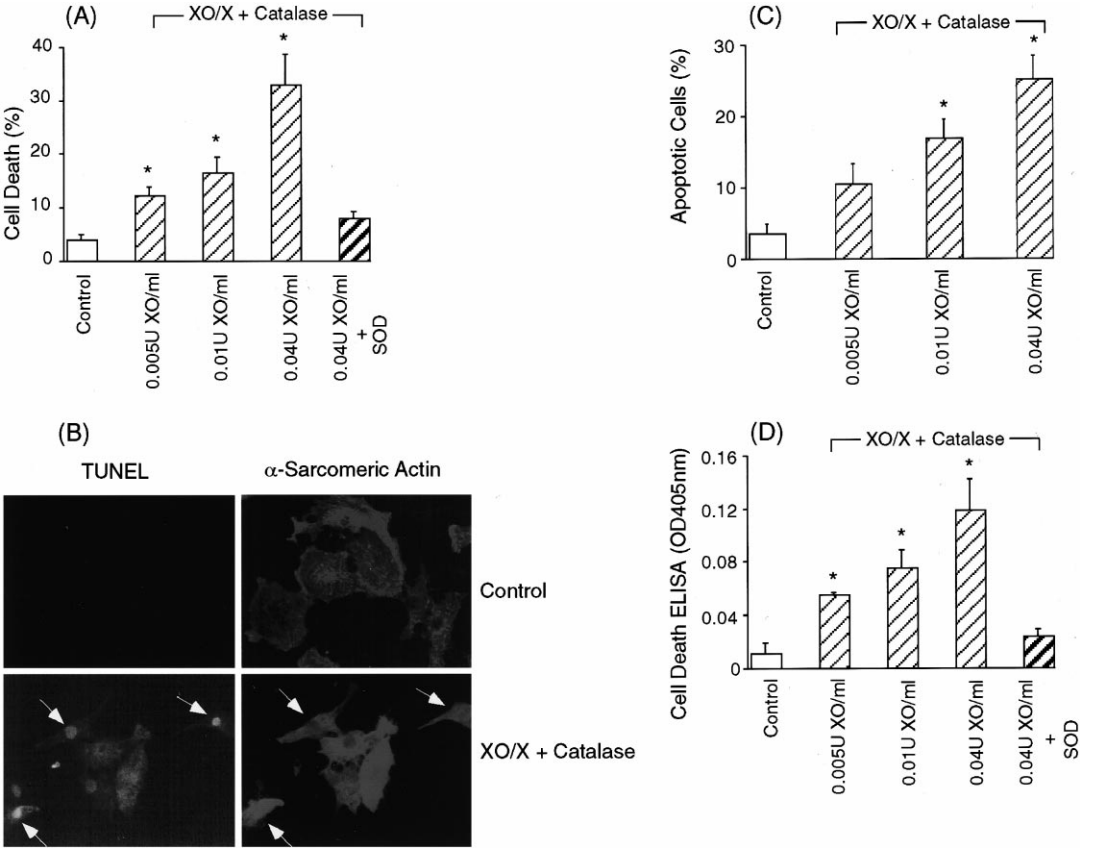

Fig. 1. O_2^- induces apoptosis in cardiac myocytes. A: Cell death was determined by trypan blue exclusion after cells were treated for 1 h with different doses of XO plus 0.1 mM xanthine in the presence of 500 U/ml catalase and further cultured for another 7 h. Superoxide dismutase (SOD) was at 1000 U/ml. *$P < 0.05$, compared to unstimulated control cells. B: Double staining of cardiac cells with TUNEL and α-sarcomeric actin. Cells were treated for 1 h with 0.04 U XO/ml plus 0.1 mM xanthine in the presence of 500 U/ml catalase and further cultured for another 7 h. TUNEL and immunostaining of cardiac myocytes were subsequently processed. Arrows indicate TUNEL-positive cardiomyocytes. Original magnification ×400. C: Quantitation of apoptotic cells detected by TUNEL. For each concentration more than 300 cells were counted within random fields and divided by the number of TUNEL positive cells. *$P < 0.05$, compared to control. D: DNA fragmentation determined by cell death ELISA. ELISA was processed after cells were exposed for 1 h to the indicated doses of XO plus 0.1 mM xanthine in the presence of 500 U/ml catalase and further cultured in medium for 7 h. Superoxide dismutase (SOD) was at 1000 U/ml. The histone-associated DNA fragments are presented as the optical density at 405 nm. *$P < 0.05$, compared to control.

antibody conjugated with alkaline phosphatase. Following two washes with 100 mM maleic acid buffer containing 0.3% Tween-20, CSPD substrate solution was added to the membrane and incubated for 10 min. The membrane was wrapped in plastic and exposed to film.

2.8. Statistical analysis

The results are expressed as means ± S.E.M. of at least three independent experiments, unless stated otherwise. Data were evaluated by Student's *t*-test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. O_2^- induces apoptosis in cardiomyocytes

XO/X was used to produce O_2^-, since XO/X yields both O_2^- and H_2O_2, the H_2O_2-scavenger catalase was employed simultaneously to decompose H_2O_2. As can be seen from Fig. 1A, XO/X in the presence of catalase could significantly induce dose-dependent increases in the percentage of dead cardiac cells. Addition of SOD almost completely prevented cell death caused by XO/X plus catalase. XO, xanthine, SOD and catalase alone had no effect on the cell death (data not shown). These data suggest that O_2^- is able to induce death in cardiomyocytes.

In order to test whether cell death caused by O_2^- occurred by apoptosis, we employed TUNEL and cell death ELISA to characterize apoptosis. As shown in Fig. 1B, after treatment with XO/X plus catalase cell nuclei were labeled by in situ (TUNEL) and identified as cardiomyocytes by staining for α-sarcomeric actin. Taking those cells as apoptotic cardiomyocytes, which simultaneously exhibited positive staining for TUNEL and α-sarcomeric actin, apoptotic cells were counted and the results are depicted in Fig. 1C. O_2^- caused dose-dependent increases in the percentage of apoptotic cells.

We next employed a cell death detection ELISA, which specifically detects histone-associated DNA-fragments within the cytoplasmic fraction of stimulated cells and which includes those cells floating in the medium of the culture dishes after treatment. As can be seen from Fig. 1D, there was a dose-dependent gradual increase of oligonucleosomes in the cytoplasmic fraction after treatment with XO/X in the presence of catalase. Administration of SOD could almost completely abrogate XO/X plus catalase-induced DNA fragmentation in cardiomyocytes. Taken together, these data suggest that O_2^- is able to induce apoptosis in cardiomyocytes.

3.2. O_2^- induces proliferation of cardiac fibroblasts

To test whether cardiac fibroblasts might also undergo apoptosis when exposed to O_2^- under the same conditions as cardiomyocytes, we first detected if cardiac fibroblasts underwent cell death after exposed to O_2^-. As illustrated in Fig. 2A, 7 h after treatment with increasing doses of XO/X in the presence of catalase there was no significant increase in the percentage of dead cells in cardiac fibroblasts. Concomitantly, the cells were negative to TUNEL, and there was no marked increase in DNA fragmentation (data not shown). These data indicate that cardiac fibroblasts do not undergo apoptosis in response to O_2^- stimulation.

In light of the distinct characteristics between cardiomyocytes and cardiac fibroblasts in which myocytes are terminally differentiated whereas fibroblasts are able to proliferate, we reasoned that fibroblasts might undergo proliferation in response to O_2^- stimulation, as we had previously observed in vascular smooth muscle cells [13]. To test this hypothesis, after treatment with O_2^- we analyzed cell proliferation by using [^{3}H]thymidine incorporation to detect DNA synthesis and by counting cell number. Exposure of cardiac fibroblasts to XO/X in the presence of catalase resulted in increases of [^{3}H]thymidine incorporation (Fig. 2B) as well as cell number (Fig. 2C), while the administration of SOD abolished XO/X plus catalase-induced increases in DNA-synthesis and cell number. XO, xanthine, SOD and catalase alone had no effect

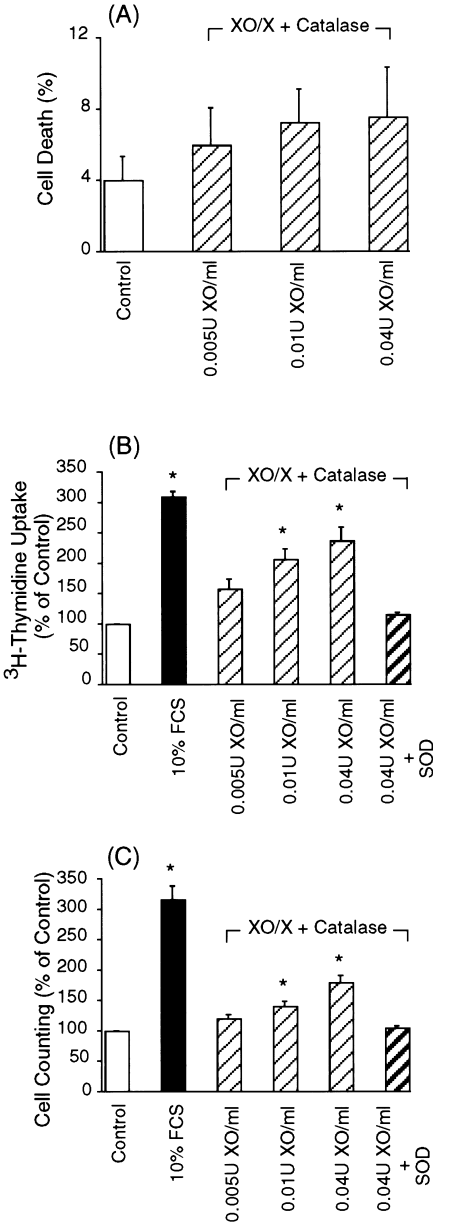

Fig. 2. Effect of O_2^- on death and proliferation of cardiac fibroblasts. A: Cell death was determined by trypan blue exclusion after cells were treated for 1 h with different doses of XO plus 0.1 mM xanthine in the presence of 500 U/ml catalase and further cultured for another 7 h in the presence of 10% fetal calf serum (FCS). B: [^{3}H]Thymidine incorporation. Cardiac fibroblasts were exposed to different doses of XO plus 0.1 mM xanthine in the presence of 500 U/ml catalase. Superoxide dismutase (SOD) was at 1000 U/ml. Cardiac fibroblasts in 10% FCS served as positive control. $^*P < 0.05$, compared to control. C: Cell counting. Cell number was counted 6 days after treatment. $^*P < 0.05$, compared to control.

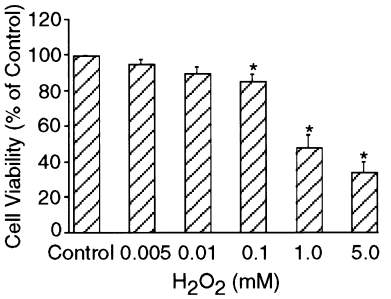

Fig. 3. Effect of H_2O_2 on the viability of cardiac fibroblasts assessed by trypan blue exclusion. Cells were treated for 1 h with different doses of H_2O_2 and further cultured in H_2O_2-free medium for 7 h. $^*P < 0.05$, compared to control.

on [^{3}H]thymidine incorporation and cell number (data not shown). Thus, cardiac fibroblasts underwent proliferation in response to O_2^- stimulation.

H_2O_2 is the product of O_2^- dismutation. To test if H_2O_2 serves as a mediator of O_2^- in the induction of cell proliferation, we exposed cardiac fibroblasts to H_2O_2 which is known to easily penetrate cell membranes. As shown in Fig. 3 H_2O_2 caused cell death rather than proliferation, excluding the possibility that H_2O_2 is involved in the signal pathway of O_2^-.

3.3. TGF-β1 is upregulated in cardiac fibroblasts in response to O_2^- stimulation

Since it has been shown that cardiac fibroblasts are able to produce TGF-β1 [8,9] a growth factor which is involved in cardiac fibrosis, this led us to test whether O_2^- can stimulate the gene expression of TGF-β1. As shown in Fig. 4, XO/X in the presence of catalase led to time-dependent increases in TGF-β1 gene expression, and such increases could be abrogated by the addition of SOD. XO, xanthine, catalase and SOD alone had no effect on TGF-β1 expression (data not shown). These data suggest that in cardiac fibroblasts TGF-β1 is upregulated upon O_2^- stimulation.

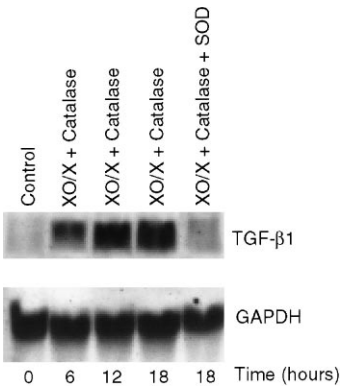

Fig. 4. Effect of O_2^- on TGF-β1 expression analyzed by Northern blot. Cardiac fibroblasts were exposed to XO at 0.04 U/ml plus 0.1 mM xanthine in the presence of 500 U/ml catalase. Superoxide dismutase (SOD) was at 1000 U/ml. Total RNA was isolated from cells at the indicated time. Relative amounts of mRNA were normalized by rat GAPDH mRNA. One representative blot from three independent experiments is shown.

4. Discussion

It appears that the O_2^--induced spectrum of cellular alterations is broad and in fact may include such divergent effects as cell growth and cell death depending largely on the afflicted cell type. This notion is supported by our present data revealing that under the same stimulation with O_2^- two types of cardiac cells behave differently with apoptosis occurring in cardiomyocytes and proliferation occurring in cardiac fibroblasts.

The present observation of the apoptotic effect of O_2^- on cardiomyocytes, a terminally differentiated cell type, is consistent with a study performed on another type of postmitotic cells, neurons, where scavenging of O_2^- by overexpression of Cu/Zn-superoxide dismutase delays apoptosis in neuronal cells deprived of nerve growth factor [14]. Also, O_2^- can mediate staurosporine-induced apoptosis of hippocampal neurons [15]. It is not yet clear how O_2^- initiates the apoptotic program. There is evidence showing that mitochondria play a pivotal role in apoptosis by releasing cytochrome *c* [16,17] or by the opening of mitochondrial membrane transition [18,19]. It is unlikely that cytochrome *c* participates in O_2^--induced cardiomyocyte apoptosis because it is not released upon O_2^- stimulation (our unpublished data). Overexpression of manganese superoxide dismutase restored mitochondrial transmembrane potential, suggesting a possible link between O_2^- and mitochondrial integrity [20]. Whether O_2^- is able to induce mitochondrial membrane transition in cardiomyocytes remains to be elucidated.

The failure of O_2^- to induce apoptosis in cardiac fibroblasts indicates the existence of cell type specific differences in the response to O_2^-. This is in agreement with other observations. For example, O_2^-, which is generated by XO/X, prevents Fas-mediated apoptosis in different tumor cell lines [21]. Vascular smooth muscle cells are stimulated to proliferate by O_2^- [13,22]. On the other hand, O_2^- can play a role in mediating growth signals. O_2^- mediates cell proliferation stimulated by lactosylceramide [23] or by serotonin [24] via activation of the kinase cascade in smooth muscle cells. However, not all mitotic cell types proliferate in response to O_2^-. Instead, O_2^- can be a mediator to trigger the execution of apoptosis in mitotic cells. O_2^- is able to convey death signals of growth factor deprivation in apoptosis of proximal tubular cells [25]. In addition, O_2^- interacts with nitric oxide to form peroxynitrite, which has been shown to induce apoptosis in several types of cells [26,27].

Although there is evidence showing that there is a link between oxidative stress and the development of heart failure [2,3], it is unclear how oxidative stress contributes to this disease. Our data provide strong evidence indicating that O_2^- could be a potential factor involved in the development of the well characterized phenotype of the failing heart consisting of cardiomyocyte death and interstitial fibrosis.

It has been shown that myocardial ischemia/reperfusion is accompanied by a significant increase in the expression TGF-β1 [28,29]. Because ischemia/reperfusion leads to the formation of O_2^-, our data that O_2^- is able to upregulate the gene expression of TGF-β1 in cardiac fibroblasts may provide a link between ischemia/reperfusion and TGF-β1 expression.

In our present work TGF-β1 was upregulated in cardiac fibroblasts by the direct stimulation with O_2^-. It would be of interest to study if O_2^- can be a signaling mediator for growth

factors like angiotensin II in stimulating the pathogenesis of myocardial fibrosis.

References

[1] Haunstetter, A. and Izumo, S. (1998) Circ. Res. 82, 1111–1129.
[2] Keith, M., Geranmayegan, A., Sole, M.J., Kurian, R., Robinson, A., Omran, A.S. and Jeejeebhoy, K.N. (1998) J. Am. Coll. Cardiol. 31, 1352–1356.
[3] Mallat, Z., Philip, I., Lebret, M., Chatel, D., Maclouf, J. and Tedgui, A. (1998) Circulation 97, 1536–1539.
[4] Li, Y., Huang, T.T., Carlson, E.J., Melov, S., Ursell, P.C., Olson, J.L., Noble, L.J., Yoshimura, M.P., Berger, C., Chan, P.H., Wallace, D.C. and Epstein, C.J. (1995) Nature Genet. 11, 376–381.
[5] Lebovitz, R., Zhang, H., Vogel, H., Cartwright, J., Dionne, L., Lu, N., Huang, S. and Matzuk, M. (1996) Proc. Natl. Acad. Sci. USA 93, 9782–9787.
[6] Border, W.A. and Noble, N.A. (1994) New Engl. J. Med. 331, 1286–1292.
[7] Philips, N., Bashey, R.I. and Jimenez, S.A. (1994) Cardiovasc. Res. 28, 1342–1347.
[8] Fisher, S.A. and Absher, M. (1995) Am. J. Physiol. 268, C910–917.
[9] Lee, A.A., Dillmann, W.H., McCulloch, A.D. and Villarreal, F.J. (1995) J. Mol. Cell. Cardiol. 27, 2347–2357.
[10] Simpson, P., McGrath, A. and Savion, S. (1982) Circ. Res. 51, 787–801.
[11] Mlodzik, K., Loffing, J., Le Hir, M. and Kaissling, B. (1995) Histochem. Cell Biol. 103, 227–236.
[12] Sudhir, K., Wilson, E., Chatterjee, K. and Ives, H.E. (1993) J. Clin. Invest. 92, 3003–3007.
[13] Li, P.F., Dietz, R. and von Harsdorf, R. (1997) Circulation 96, 3602–3609.
[14] Greenlund, L.J., Deckwerth, T.L. and Johnson Jr., E. (1995) Neuron 14, 303–315.
[15] Krohn, A.J., Preis, E. and Prehn, J.H. (1998) J. Neurosci. 18, 8186–8197.
[16] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cell 91, 479–489.
[17] Skulachev, V.P. (1998) FEBS Lett. 423, 275–280.
[18] Skulachev, V.P. (1996) FEBS Lett. 397, 7–10.
[19] Kroemer, G., Dallaporta, B. and Resche-Rigon, M. (1998) Annu. Rev. Physiol. 60, 619–642.
[20] Majima, H.J., Oberley, T.D., Furukawa, K., Mattson, M.P., Yen, H.C., Szweda, L.I. and St Clair, D.K. (1998) J. Biol. Chem. 273, 8217–8224.
[21] Clement, M.V. and Stamenkovic, I. (1996) EMBO J. 15, 216–225.
[22] Rao, G.N. and Berk, B.C. (1992) Circ. Res. 70, 593–599.
[23] Bhunia, A.K., Han, H., Snowden, A. and Chatterjee, S. (1997) J. Biol. Chem. 272, 15642–15649.
[24] Lee, S.L., Wang, W.W. and Fanburg, B.L. (1998) Free Radical Biol. Med. 24, 855–858.
[25] Lieberthal, W., Triaca, V., Koh, J.S., Pagano, P.J. and Levine, J.S. (1998) Am. J. Physiol. 275, F691–702.
[26] Lin, K.T., Xue, J.Y., Lin, M.C., Spokas, E.G., Sun, F.F. and Wong, P.Y. (1998) Am. J. Physiol. 274, C855–860.
[27] Szabo, C. and Ohshima, H. (1997) Nitric Oxide 1, 373–385.
[28] Chandrasekar, B., Streitman, J.E., Colston, J.T. and Freeman, G.L. (1998) Biochim. Biophys. Acta 1406, 91–106.
[29] Herskowitz, A., Choi, S., Ansari, A.A. and Wesselingh, S. (1995) Am. J. Pathol. 146, 419–428.