Preliminary characterisation of mechanoresponsive regions of the c-*fos* promoter in bone cells

Matthew A. Peake, Alicia J. El Haj*

Centre for Science and Technology in Medicine, School of Medicine, Keele University, North Staffordshire Hospital, Hartshill, Stoke-on-Trent, Staffordshire ST4 7QB, UK

Received 22 January 2003; accepted 24 January 2003

First published online 6 February 2003

Edited by Veli-Pekka Lehto

Abstract Preliminary analysis of the mechanisms involved in induction of stretch-mediated transcriptional activity in the c-*fos* promoter of bone has been undertaken using a series of c-*fos* promoter–reporter constructs. UMR-106 osteoblastic cells transfected with reporter constructs were subjected to cyclical physiological loading. The major determinants in the resulting transcriptional mechanoactivation are within the sequence between −356 and −151 which contains the serum response element and a consensus shear stress response element. Elements beyond this region also play a role as deletion of this region does not eliminate mechanoinduction. These results suggest that the mechanical induction of c-*fos* in osteoblastic bone cells is mediated by multiple response elements.

Key words: Mechanical stretch; c-*fos*; Promoter; Bone; Shear stress response element

1. Introduction

Mechanically induced signalling cascades are known to target particular components of the AP-1 transcription factor complex, namely Fos and Jun [1], and may play a critical role in differential control of transcription mediated by AP-1. The role of AP-1 in the mechanoinduction of gene expression has recently been demonstrated in bone [2].

The c-*fos* promoter has been demonstrated to be responsive to a wide variety of stimuli, which include serum [3], KCl [4], parathyroid hormone (PTH) [5], ATP [6], prostaglandin E_2 (PGE_2) [7], and mechanical forces (in cardiac myocytes) [8–11]. The major response elements of the mouse c-*fos* promoter have been delineated and are present within the 400 bp region located directly upstream of the transcriptional start site. These are the serum response element (SRE), calcium/cAMP response element(s) (CREs) and the *sis*-inducible element (SIE) (Fig. 1). Investigation of the role of specific elements within the c-*fos* promoter has been conducted in other, non-bone, cell types. However, the ways in which hormones and mechanical cues may influence these elements may potentially vary between tissue types. Despite extensive investigations into the c-*fos* promoter in other cell types, the corresponding studies in osteoblasts have so far been predominantly limited to the action of PTH [5,12,13] with some studies in osteoblastic cell lines including PGE_2 [7], ATP [6], epidermal growth factor and phorbol ester [5,13].

Recently, we have investigated the signalling cascades involved in mechanical induction of c-*fos* up-regulation in bone and demonstrated the potential role of calcium- and integrin-mediated downstream cascades [14]. Ca^{2+} influxes in bone cells are known to be induced by cyclic loading [15–17] and are thought to be involved in the mechanical induction of c-*fos* expression [14]. Calcium-mediated pathways are able to target both SRE and CRE elements, whereas alternative pathways, e.g. those mediated by extracellular signal-regulated kinase or cAMP, target single response elements (the SRE and CRE respectively [1,12]).

Previous in vitro and in vivo studies in non-osteoblastic cell types (i.e. cardiac myocytes) have indicated that mechanoinduction may be mediated by a single response element, namely the SRE [10]. The presence and role of a second mechanoresponsive promoter element, the shear stress response element (SSRE), has been defined in non-bone cells, with the SSRE being implicated in the mechanical induction of platelet-derived growth factor (PDGF) gene expression resulting from shear stress in endothelial cells [18,19]. More recently the SSRE has been hypothesised to be involved in the stretch-activated induction of extracellular matrix proteins tenascin-C and collagen XII [20]. The SSRE sequence is also present within the c-*fos* promoter (Fig. 1) and, although no role in mechanoinduction has been identified so far in c-*fos*, this will be examined.

In this study, analysis of sequential deletions of the c-*fos* promoter and a promoter construct carrying a specific base pair mutation (within the major CRE) will be undertaken with the aim of delineating the important and essential components of the c-*fos* promoter required for any transcription-mediated mechanoresponse.

2. Materials and methods

2.1. Cell culture

Rat osteosarcoma UMR-106 cells [21] (a gift from Prof. T.J. Martin, University of Melbourne, Australia) were cultured in α-minimal essential medium (α-MEM) supplemented with 10% foetal calf serum (FCS) and 1% dilution of 100× antibiotic/antimycotic solution (1×AB). Confluent cultures were treated with 1×trypsin-EDTA (Sigma-Aldrich, Gillingham, Dorset, UK) and passaged onto collagen I-coated coverslips prior to transfection and subsequent mechanical loading.

2.2. Coverslip coating protocol

Glass coverslips were coated with collagen type I (from bovine calf skin) as previously described [14]. Briefly, the central 24×30 mm

*Corresponding author. Fax: (44)-1782-717079.
E-mail address: bea17@keele.ac.uk (A.J. El Haj).

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00108-X

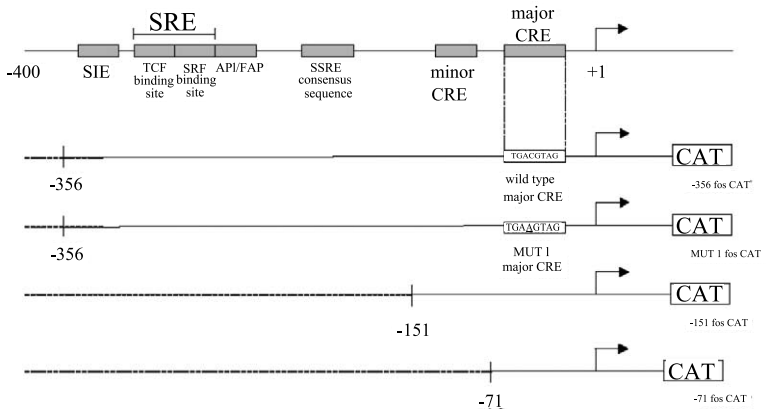

Fig. 1. A schematic diagram of the mouse c-*fos*-CAT promoter promoter constructs used in this study. All base pair positions quoted are relative to the transcriptional start site. SRE = serum response element; CRE = Ca^{2+}/cAMP response element; SSRE = shear stress response element; SIE = sis-inducible element; FAP = Fos-AP-1 binding sequence.

region of 24×50 mm coverslips (thickness 2; Philip Harris Scientific, Macclesfield, Cheshire, UK) were coated by adsorption of collagen type I (Sigma-Aldrich) at room temperature for 2–4 h at a concentration of 7.5 μg/cm^2. UMR-106 cells were subcultured onto coated coverslips in 500 μl droplet cultures containing 7.5×10^5 cells and 1.5×10^5 cells for reverse transcription-polymerase chain reaction (RT-PCR) and promoter-based loading studies respectively. Droplet cultures were placed on the coated portion of coverslips and cells allowed to attach overnight. Subsequently, coverslip cultures were submerged in supplemented α-MEM and cultured until the required confluence for transfection and/or mechanical loading was reached.

2.3. Plasmids

The mouse c-*fos* promoter constructs utilised in this study contain the major response elements thought to be involved in the transcriptional response to those factors detailed above (see Fig. 1). The mouse c-*fos* promoter plasmid constructs (a gift from Prof. N.C. Partridge, University of Medicine and Dentistry of New Jersey, NJ, USA) represent the proximal 5′ region of the promoter from −356, −151 and −71 upstream of the transcriptional start site to a point downstream of the start site at +109 [22]. In each of these cases, the functional promoter sequence was located directly upstream of the coding sequence for the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. In addition to the deletion constructs mentioned, a mutant mouse c-*fos* promoter plasmid, Mut 1 [23], which contains a single base pair mutation in the −60 major CRE, was utilised (Fig. 1). In addition to the c-*fos* promoter plasmids, two control plasmids were utilised, namely RSV CAT (a gift from Dr S. Frost, University of Keele, Staffordshire, UK) and pCMV SPORT β-galactosidase (β-gal; Invitrogen, Paisley, Renfrewshire, UK), which were used to assess non-c-*fos*-regulated (control) CAT expression and transfection efficiency respectively.

2.4. Transient transfection

Transient co-transfection of UMR-106 cells with c-*fos*- or RSV-CAT and pCMV SPORT β-gal control plasmids was carried out with transfection of cells taking place directly on the coverslip. Transfection was preceded by the seeding of 1.5×10^5 cells to each collagen type I-coated coverslip for 2 days to reach the full confluence. 4 μg of CAT plasmid DNA and 4 μg of β-gal plasmid DNA were then co-transfected into each coverslip culture using 8 μl of Superfect (Qiagen, Crawley, West Sussex, UK) following the manufacturer's supplied protocol. The transfection solution was added directly to the coverslip culture and incubated in a normal 5% CO_2 incubator at 37°C for 2–3 h. Following removal of the transfection solution, cultures were washed with sterile phosphate-buffered saline, and incubated overnight with α-MEM supplemented with 10% FCS and 1×AB. The culture medium was then replaced with α-MEM supplemented with 2% FCS (and 1×AB). Experimental treatments of transfected cultures, with chemical or mechanical stimulation, were run in parallel with 2% FCS-treated control cultures.

2.5. Loading protocol

The four-point loading system was used to apply cyclical load experimentally to sets of four collagen I-coated coverslips seeded with UMR-106 bone cells. Using methodology described previously [14,23], coverslip cultures were mechanically loaded at a strain of ∼1000 μstrains. Loading was applied homogeneously across the coverslip culture area at a 1 Hz frequency for 1800 cycles, equivalent to 30 min. Control coverslip cultures were prepared in parallel using an identical procedure, without loading.

For plasmid-based promoter analysis studies, both control and mechanically loaded UMR-106-transfected coverslip cultures were lysed 6 h post-load by the addition of 1×CAT ELISA cell lysis buffer (Roche Diagnostics Ltd, Lewes, East Sussex, UK), with treated cultures being incubated at room temperature for 30 min. Promoter activity in control and mechanically loaded cultures was assessed by quantification of CAT and β-gal protein expression using ELISA.

2.6. RT-PCR analysis

For RT-PCR-based studies, loaded and parallel unloaded coverslip cultures were harvested/lysed in modified guanidinium thiocyanate solution 1 h after the completion of loading and total RNA was extracted using a modification of the guanidinium thiocyanate method [24] as described by Gu et al. [25]. Total RNA from each control and loaded coverslip culture was used as a template for reverse transcription with 200 U of Superscript II reverse transcriptase (Invitrogen) using random hexamer oligonucleotide primers (Invitrogen) to a final concentration of 12.5 ng/μl. Initial mRNA denaturation at 70°C for 10 min preceded pre-incubation at 25°C for 10 min followed by incubation and reverse transcription at 42°C for 50 min. Following reverse transcriptase inactivation at 70°C for 15 min, 1 μl of the reaction products from each sample was used as a PCR template. PCR amplification reactions were carried out using primer pairs specific for human c-*fos*, and HPRT (control) cDNA respectively. Primer sequences were as follows: c-*fos* forward primer: 5′-TCT-CTT-ACT-ACC-ACT-CAC-CC-3′; c-*fos* reverse primer: 5′-TGG-AGT-GTA-TCA-GTC-AGC-TC-3′; HPRT forward primer: 5′-TTG-TAG-CCC-TCT-GTG-TGC-TCA-AG-3′; HPRT reverse primer 5′-GCC-TGA-CCA-AGG-AAA-GCA-AAG-TC-3′. Amplification of both c-*fos* and HPRT utilised 40 cycles of amplification, which consisted of incubation at 95°C, 55°C, and 72°C for 45 s each. In each case, amplification was carried out using 0.5 U of Taq polymerase (Promega UK, Southampton, UK) and all primers were used at a 0.5 μM final concentration.

Following completion of the PCR, samples were separated by electrophoresis on a 1% agarose in 1×TBE gel at 100 V for 60 min. The resulting gel was then visualised under UV illumination and images were captured using a digital camera-based gel documentation system (Imagestore 5000, Ultra-Violet Products, Cambridge, UK).

2.7. CAT and β-gal expression analysis

Quantification of CAT and β-gal protein levels was carried out following equilibration of the amounts of total protein present in

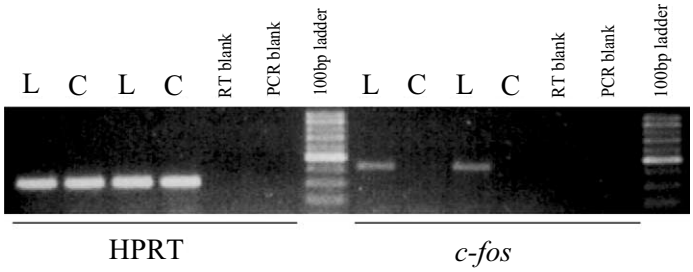

Fig. 2. Mechanical induction of c-*fos* gene expression in UMR-106 cells. Total RNA extracted from UMR-106 coverslip cultures 1 h after a 30 min period of mechanical loading of ~1000 μstrains at 1 Hz was used as a template for RT-PCR analysis using primers specific for c-*fos* and HPRT. C = control L = loaded.

each control and test sample. Total protein levels were determined using a total protein assay kit (Sigma-Aldrich) as per the manufacturer's supplied protocols. Quantification of CAT and β-gal protein levels was carried out separately using CAT ELISA and β-gal ELISA kits respectively (Roche Diagnostics). Colorimetric analysis of standard and test sample wells was carried out using a Dynatech MR5000 plate reader.

3. Results

3.1. RT-PCR analysis

RT-PCR analysis of c-*fos* cDNA in control (non-loaded) and loaded UMR-106 collagen I coverslip cultures resulted in the amplification of a single 417 bp fragment in loaded samples only (Fig. 2). Parallel RT-PCR analysis showed similar levels of the housekeeping gene HPRT in all control and loaded samples (Fig. 2). This transcriptional up-regulation of c-*fos* was observed at 1 h post load.

3.2. Mechanical induction of the mouse c-fos promoter

Mechanical load applied to osteoblastic cells transfected with the 5′ regions of the c-*fos* promoter (Fig. 3) induced an increase in CAT protein expression using the 'full-length' −356 plasmid. This almost 100% increase was significantly reduced by loss of the region stretching from −356 to −151, with a significantly reduced load induction taking place in −151 transfected cultures. Further deletion of the promoter to the −71 position did however completely abolish the mechanical load response in promoter activation (Fig. 3). Expression from both (constitutively expressed) RSV CAT control (Fig. 3) and a co-transfected control plasmid, pCMV SPORT β-gal was found to be unaltered in response to mechanical stimulation of transfected cultures (data not shown).

Mechanical loading of cells transfected with Mut 1 plasmid resulted in a significant elevation in expression of CAT protein (Fig. 3). This response was approximately 160% of that observed in control cultures, indicating that although interaction between CREB and the major CRE at −60 was prevented, a significant level of mechanical induction was still possible. Comparison of this mechanical induction with that observed from the wild type (−356) plasmid (Fig. 3) would appear to indicate that although the levels of induction were reduced as compared to the wild type, this difference was not significant. This would appear to suggest that this mutation does not have a significant effect upon mechanoinduction of expression of the reporter gene located downstream.

4. Discussion

The results presented here demonstrate, for the first time, activation of the (mouse) c-*fos* promoter by a mechanical stimulus in osteoblastic (UMR-106) cells. This induction of the c-*fos* promoter is paralleled by induction of c-*fos* transcription with the resultant increases in c-*fos* mRNA levels detectable by RT-PCR. We have previously demonstrated a similar mechanical induction of c-*fos* at this time point in human primary bone cells and MG-63 cells using identical loading techniques by RT-PCR [14,23], quantitative RT-PCR and Northern analysis (data not shown).

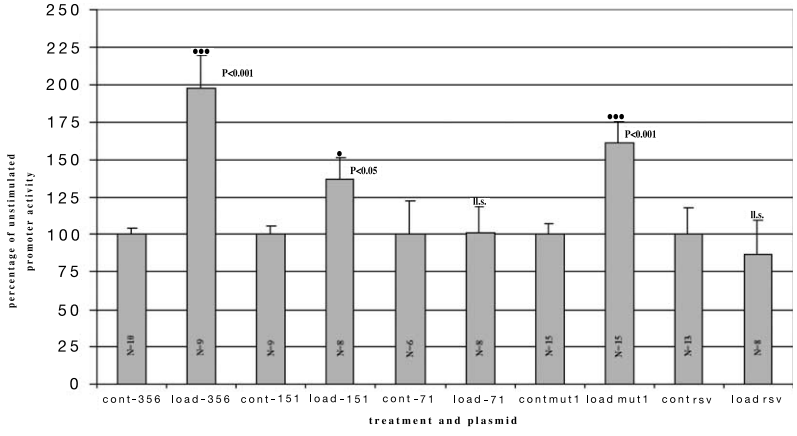

Fig. 3. Effect of mechanical load on c-*fos* promoter reporter gene expression in UMR-106 rat osteoblastic cells. Promoter activity was assessed by CAT ELISA analysis 6 h after a 30 min period of mechanical load of ~1000 μstrains at 1 Hz. $*P < 0.05$, $***P < 0.001$, n.s., not statistically significant; statistical significance based on ANOVA.

Induction of the promoter was shown to be mediated by a shortened c-*fos* promoter construct representing only 356 bp of sequence upstream of the transcriptional start site. This indicates that although other response elements may be involved in the regulation of this response in the full-length c-*fos* gene in vivo, this region is sufficient in isolation in vitro. This finding agrees with previous findings of Sadoshima et al. [9,10], who showed a similar load activation of c-*fos* promoter in cardiac myocytes.

Although the loss of the important motifs SIE, SRE, Fos-AP-1 binding sequence (FAP) and SSRE reduces the load response, the remaining fragment containing two Ca/CRE motifs is sufficient to support a highly reduced (yet still significant) mechanoinduction in UMR-106 cells. Further deletion of the promoter indicates that although the presence of the two CRE motif-containing region is sufficient to support the load response, removal of the minor CRE-containing fragment results in a loss of the response. These results are in contrast to the work of Sadoshima et al. [10] and Aoyagi and Izumo [11], which indicated that in cardiac cells both in vitro and in vivo, the SRE alone was responsible for mechanoinduction. The lack of significant suppression of the load response associated with the mutations present in the Mut 1 plasmid in addition to the deletion analysis results would appear to indicate that the major CRE is neither essential nor sufficient to orchestrate a c-*fos* response to mechanical load. This is in contrast to the induction of c-*fos* via alternative bone-related signalling pathways, namely PGE_2 [7] and PTH [12].

The dramatic and highly significant negative effect of deletion of the region −356 to −151 suggests that this region contains the response elements that are most important for the maximal mechanical induction of c-*fos*. As the major response element lost by deletion of this region of the mouse c-*fos* promoter, the SRE would appear to be a prime candidate for the pivotal role in this induction. Additional regulatory elements within this region are the SIE, FAP/AP-1 and 'SSRE' motifs. Although a role for the SRE in the load induction of c-*fos* has been demonstrated in other cell types [9–11], the other response elements, particularly the SSRE, may also play a role. Mechanically inducible SSRE sequences have been observed in the regulatory sequences of multiple genes including PDGF [18], collagen XII [20] and tenascin-C [26]. As the SSRE of c-*fos* is contained within the promoter fragment responsible for the greatest level of mechanoinduction, further work to examine the exact role of the SSRE in c-*fos* mechanical induction in bone is desirable.

Instead of the discovery of a single mechanoresponse element, a more complex and interacting picture is emerging. It appears that in the case of the mechanical stimulation of osteoblasts, multiple elements are required to induce a complete and maximal transcriptional response. This is in contrast to work on c-*fos* mechanical activation in cardiac myocytes, which requires only the SRE region to be present [10]. The utilisation of additional promoter elements in the case of osteoblastic cells may be due to the utilisation of additional and/or alternative pathways in this cell type which cannot be mediated via the SRE. For example, the activation of c-*fos* by cAMP-mediated pathways would be possible via one or more of the CREs present. The involvement of a CRE has been demonstrated in the mechanoactivation of *cox-2* expression in osteoblasts [2].

Further possible reasons for these differences in promoter element usage may include the mode of mechanical stimulation, which differs slightly between each of these studies. In the studies of cardiac myocytes, cells were mechanically stimulated by hypotonic stress-induced cell swelling [10]. This system differs from the four-point bending apparatus used in this study and may be a factor in the observed differences between cell types. For example, mechanoactivation of c-*fos* differs in each of these systems with respect to their requirement for calcium signalling pathways as demonstrated by the inhibition of c-*fos* mechanoinduction in osteoblasts following EGTA treatment. Treatment of cardiac myocytes with EGTA fails to inhibit c-*fos* induction by hypotonic stress [27]. Further work is needed to define more closely the specific elements involved.

References

[1] Hipskind, R.A. and Bilbe, G. (1998) Front. Biosci. 3, D804–D816.
[2] Ogasawara, A., Arakawa, T., Kaneda, T., Takuma, T., Sato, T., Kaneko, H., Kumegawa, M. and Hakeda, Y. (2001) J. Biol. Chem. 276, 7048–7054.
[3] Treisman, R. (1985) Cell 42, 889–890.
[4] Johnson, C.M., Hill, C.S., Chawla, S., Treisman, R. and Bading, H. (1997) J. Neurosci. 17, 6189–6202.
[5] Evans, D.B., Hipskind, R.A. and Bilbe, G. (1996) J. Bone Miner. Res. 11, 1066–1074.
[6] Bowler, W.B., Dixon, C.J., Halleux, C., Maier, R., Bilbe, G., Fraser, W.D., Gallagher, J.A. and Hipskind, R.A. (1999) J. Biol. Chem. 274, 14315–14324.
[7] Fitzgerald, J., Dietz, T.J. and Hughes-Fulford, M. (2000) Endocrinology 141, 291–298.
[8] Komuro, I., Katoh, Y., Kaida, T., Shibazaki, Y., Kurabayashi, M., Hoh, E. and Takaku, F. (1991) J. Biol. Chem. 266, 1265–1268.
[9] Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T.J. and Izumo, S. (1992) J. Biol. Chem. 267, 10551–10560.
[10] Sadoshima, J., Qiu, Z., Morgan, J.P. and Izumo, S. (1996) EMBO J. 15, 5535–5546.
[11] Aoyagi, T. and Izumo, S. (1993) J. Biol. Chem. 268, 27176–27179.
[12] Pearman, A.T., Chou, W.Y., Bergman, K.D., Pulumati, M.R. and Partridge, N.C. (1996) J. Biol. Chem. 271, 25715–25721.
[13] Fluhmann, B., Zimmermann, U., Muff, R., Bilbe, G., Fischer, J.A. and Born, W. (1998) Mol. Cell. Endocrinol. 139, 89–98.
[14] Peake, M.A., Cooling, L.M., Magnay, J.L., Thomas, P.B. and El Haj, A.J. (2000) J. Appl. Physiol. 89, 2498–2507.
[15] Pommerenke, H., Schreiber, E., Durr, F., Nebe, B., Hahnel, C., Moller, W. and Rychly, J. (1996) Eur. J. Cell Biol. 70, 157–164.
[16] El Haj, A.J., Walker, L.M., Preston, M.R. and Publicover, S.J. (1999) Med. Biol. Eng. Comput. 37, 403–409.
[17] Walker, L.M., Publicover, S.J., Preston, M.R., Said Ahmed, M.A. and El Haj, A.J. (2000) J. Cell Biochem. 79, 648–661.
[18] Resnick, N. and Gimbrone Jr., M.A. (1995) FASEB J. 9, 874–882.
[19] Khachigian, L.M., Resnick, N., Gimbrone Jr., M.A. and Collins, T. (1995) J. Clin. Invest. 96, 1169–1175.
[20] Chiquet, M. (1999) Matrix Biol. 18, 417–426.
[21] Martin, T.J., Ingleton, P.M., Underwood, J.C., Michelangeli, V.P., Hunt, N.H. and Melick, R.A. (1976) Nature 260, 436–438.
[22] Lee, G. and Gilman, M. (1994) Mol. Cell. Biol. 14, 4579–4587.
[23] Peake, M., Yang, Y., Cooling, L. and El Haj, A.J. (2000) Proc. Mechanotransduct. 2000, 177–184.
[24] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[25] Gu, Y., Preston, M.R., el Haj, A.J., Hamid, J., Zamponi, G.W., Howl, J. and Publicover, S.J. (1999) Pflugers Arch. 438, 553–560.
[26] Chiquet-Ehrismann, R., Tannheimer, M., Koch, M., Brunner, A., Spring, J., Martin, D., Baumgartner, S. and Chiquet, M. (1994) J. Cell Biol. 127, 2093–2101.
[27] Sadoshima, J. and Izumo, S. (1997) Heart Vessels 12 (Suppl.), 194–197.