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Nitric Oxide 12 (2005) 89–96
NITRIC OXIDE Biology and Chemistry
www.elsevier.com/locate/yniox

Extracorporeal shock waves: From lithotripsy to anti-inflammatory action by NO production

Sofia Mariotto [a,1], Elisabetta Cavalieri [a,1], Ernesto Amelio [b], Anna Rosa Ciampa [a], Alessandra Carcereri de Prati [a], Ernst Marlinghaus [c], Sergio Russo [d], Hisanori Suzuki [a,*]

[a] Department of Neuroscience and Vision, Section of Biochemistry, University of Verona, Strada Le Grazie 8, 37134 Verona, Italy
[b] Shock Wave Unit, Hand Surgery Department, Policlinic G.B. Rossi, Largo L.A. Scuro 10, 37134 Verona, Italy
[c] Applied Research Center, Storz Medical AG, Kreuzlingen, Switzerland
[d] Department of Orthopaedic, Policlinic Federico II, University of Naples, Via S. Pansini 5, 80131 Naples, Italy

Received 7 June 2004; revised 30 November 2004

Abstract

At low energy density (0.03 mJ/mm^2), extracorporeal shock waves (ESW), originally developed for clinical lithotripsy, have successfully been used for anti-inflammatory treatment of soft tissues. Since nitric oxide plays a critical role in inflammation, we hypothesized for ESW to increase NO production in cells. Using human umbilical vein endothelial cells as a model system, we observed that ESW, at low energy density, rapidly induced an enhancement of eNOS activity. In these cells, eNOS activity is modulated by tyrosine- and serine-phosphorylation. ESW shifted eNOS to a less-tyrosine-phosphorylated form, without affecting its serine-phosphorylation, thus accounting for its rapid enzyme activation. LPS/IFN-γ treatment of human umbilical vein endothelial cells induced a rapid inhibition of eNOS activity and concomitant NF-κB activation which were efficiently counteracted by ESW treatment. Therefore, the present results indicate that the molecular mechanism of clinically observed anti-inflammatory action of ESW should include tyrosine-dephosphorylation of eNOS, a successive increase in NO production and suppression of NF-κB activation.

Keywords: Shock waves; Nitric oxide; Inflammation; HUVEC; Endothelial nitric oxide synthase

Shock waves (SW), defined as a sequence of single sonic pulses characterized by high peak pressure (100 MPa), fast pressure rise (<10 ns), and short lifecycle (10 μs), are conveyed by an appropriate generator to a specific target area with the energy density in the range of 0.003–0.890 mJ/mm^2.

Extracorporeal SW (ESW) therapy was first applied in patients in 1980 to break up kidney stones [1]. During the last 10 years, this technique has been successfully employed in orthopaedic diseases [2] such as pseudoarthosis [3], tendinitis calcarea of the shoulder [4,5], epicondylitis [6], plantar fasciitis [7], and several inflammatory tendon diseases. In particular, treatment of the tendon and muscle tissues was found to induce a long-time (1–4 months) tissue regeneration effect [8] besides a more immediate anthalgic and anti-inflammatory outcome. Moreover, an increase of neoangiogenesis in the tendons of dogs was observed after 4–8 weeks of ESW treatment [9]. Although the biochemical mechanisms underlying these effects have to be elucidated yet, clinical observations indicate an immediate increase in blood flow around the treated area.

In the attempt to identify the molecular mechanism of ESW anti-inflammatory action, we hypothesized

* Corresponding author. Fax: +39 045 8027170.
E-mail address: hisanori.suzuki@univr.it (H. Suzuki).
[1] These two authors contributed equally to this work.

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doi:10.1016/j.niox.2004.12.005

that nitric oxide (NO) plays a critical role in this therapeutic action, based on the following considerations: (i) NO is ubiquitously produced by NO synthase (NOS); (ii) NO is mainly involved in vasodilatation, by being one of the most powerful vasodilators known up to date; (iii) NO is critically involved in neoangiogenesis; and (iv) physiological level of NO regulates inflammatory events, acting at the early phase of inflammation, by down-regulating either the activation of the nuclear factor NF-κB [10–12] or the catalytic activity of constitutive NOSs, especially neuron one [13–15]. Inhibition of NF-κB activation may successively down-regulate the expression of genes playing a critical role in inflammation, including inducible NOS (iNOS). Direct inhibition of iNOS catalytic activity elicited by NO [16,17] may further contribute to complete the negative feedback loop.

Three different NOS isoforms synthesize NO through sequential oxidation of L-arginine into L-citrulline using NADPH, FAD, and tetrahydrobiopterine for the catalytic activity [18,19]. Two isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS), are constitutively expressed and require, other than those described above, Ca^{2+}/calmodulin for their enzymatic activity. The third one, iNOS, is expressed in response to inflammatory mediators and it is Ca^{2+}/calmodulin-independent.

eNOS, mainly present in endothelial cells, exists either in strict contact with other proteins such as calmodulin [20], caveolin 1–3 [21], and heat shock protein 90 (Hsp90) [22] or in variously phosphorylated states [21–23]. Any condition modifying these situations may lead to the change in the amounts of NO produced by eNOS. This enzyme is primarily phosphorylated on serine residues and to a less extent on threonine and tyrosine residues. The serine-phosphorylation by phosphoinositide-3-kinase (PI3K) and protein kinase B (Akt) is a topic of intense current investigation, resulting in the modulation of eNOS enzymatic activity [24–27]. On the other hand, the precise role of tyrosine-phosphorylation of eNOS remains to be elucidated [28–30]. Furthermore, as described in recent reports, physical forces such as shear stress may induce a drastic change in cytoscheletal structure of endothelial cells [31] as well as an enhancement of eNOS activity by phosphorylation [28,32].

In the present study, we examined the possibility that ESW modulate NO production in human umbilical vein endothelial cells (HUVEC) either under normal or inflammatory conditions. We present data showing that ESW rapidly enhances eNOS activity in these cells by shifting the balance between the native and phosphorylated states to a less-tyrosine-phosphorylated one. This suggests that, under acute inflammatory conditions, increased NO level and successive suppression of NF-κB activation account, at least partly, for clinically beneficial action on tissue inflammation.

Experimental procedures

HUVEC cultures

Human umbilical vein endothelial cells were isolated from human umbilical cords collected within a few hours after spontaneous delivery by using 0.2% collagenase A (Roche Diagnostics) according to the procedure described by Jaffe et al. [33]. Cells were grown on 0.2% gelatin-coated tissue culture plates in M199 endothelial growth medium with Earle's salts (Sigma) supplemented with 20% FBS, 2 mM glutamine, 5 UI/ml heparin, 50 μg/ml ECGS (Sigma), 100 UI/ml penicillin, and 100 μg/ml streptomycin, in humidified atmosphere of 95% air, 5% CO_2 at 37 °C.

HUVEC were characterized by immunofluorescence for Von Willebrand factor and by microscope observation of typical cobblestone morphology. Cells used in all the experiments were passaged two to four times.

ESW treatment

An electromagnetic lithotriptor (MODULITH SLK device Storz Medical AG, Swizerland) was used throughout the present study. For eNOS enzymatic activity, 4×10^6 cells were harvested and suspended in 2 ml serum free medium. In these experiments, the instrument was equipped with a support device for Greiner 2-ml test tubes, to allow the correct alignment of the focal point with the test tube.

All samples, treated and not treated ones, were thermally regulated at 37 °C. For the enzyme activity assay, vials were cooled in ice after the end of the treatment and kept at −80 °C until processed. Otherwise, for the electrophoretic mobility shift assay (EMSA) cells in petri dishes were treated with ESW directly focusing the centre of the plate under ecographic control and thereafter were maintained for 30 min in humidified atmosphere of 95% air plus 5% CO_2 at 37 °C.

To measure NO production, petri plates, containing cells on glass coverslips, were treated directly focusing shock waves in the centre of the plate under ecographic control.

eNOS assay

NOS activity was estimated by measuring the conversion of L-2,3,4,5-[^{3}H]arginine to L-2,3-[^{3}H]citrulline according to the modification of the method described by Bredt and Snyder [34]. Briefly, 4×10^6 HUVEC were lysed by freezing and thawing method in 100 μl of a buffer containing 50 mM Hepes, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml antipain, and 1 mM phenylmethylsulphonyl fluoride. After centrifugation (100,000*g*, 30 min at 4 °C), the pellet was washed twice

with lysis buffer and then solubilized with 20 mM Chaps. An aliquot of the supernatant, obtained after centrifugation (100,000*g*, 30 min at 4 °C) of the solubilized pellet, was added to a reaction mixture of a final volume of 100 µl containing 50 mM Hepes, pH 7.4, 20 nM [^{3}H]arginine, 1 µM arginine, 1 mM NADPH, 1 mM EDTA, 1.2 mM $CaCl_2$, 1 µg/ml calmodulin, 10 µM FAD, 0.1 mM (6*R*)-5,6,7,8-tetrahydro-1-biopterin, and 1 mM dithiothreitol. The reactions were stopped by adding 0.4 ml (1:1) of slurry of Dowex AG50WX-8, Na^+ form (Bio-Rad), in 50 mM Hepes, pH 5.5, and after 15 min of shaking, radioactivity in the supernatant was measured. The enzyme activity was linear up to 15 min of incubation. Protein concentration in the samples was determined by the method of Bradford [35].

DAF-2DA method

Intracellular NO production was determined using specific cell permeable fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA; Alexis) and confocal microscopy. For confocal measurement, HUVEC were plated on glass coverslips and grown to sub-confluence. Ten micromolar of DAF-2DA was added to the cells cultured in serum free medium and incubated at 37 °C for 10 min. DAF-2, produced by cytosolic esterases, was converted in the presence of NO and O_2 in fluorescent triazole derivative, DAF-2T (l Ex. 492 nm; l Em. 515 nm) [36,37]. Cells were washed with PBS twice, then fixed with 3% paraformaldehyde. The samples were embedded in 0.01% DABCO and analysed using confocal laser scanning microscope (Axioplan 2, LSM 510, Carl Zeiss, Göttingen, Germany) equipped with argon (488 nm). The laser intensity, the shutter aperture, and the exposure/integration settings were kept constant to allow quantitative comparisons of relative fluorescence intensity of cells between treated groups. Images were digitally acquired and processed for fluorescence determination at the single-cell level on a Macintosh 6100/66 computer, using the NIH Image 1.61 program.

Electrophoretic mobility shift assay

Nuclear extracts of HUVEC were prepared according to Osborn et al. [38] in the presence of 10 µg/ml leupeptin, 5 µg/ml antipain, 5 µg/ml pepstatin, and 1 mM phenylmethylsulphonyl fluoride. Eight micrograms of nuclear extract was incubated with 2–5 × 10^4 cpm of ^{32}P-labeled double-stranded oligonucleotides, containing the consensus NF-κB DNA-binding site (5′-GATCCA GAGGGGACTTTCCGAGTAC-3′) of the interleukin-6 promoter, in a 15 µl reaction mixture containing 20 mM Hepes, pH 7.9, 50 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 2 µg of poly(dI–dC), 1 µg of salmon sperm DNA, and 10% glycerol. Incubation time was 15 min at room temperature. Products were fractionated on a non-denaturing 5% polyacrylamide gel.

Results

Effect of ESW on eNOS activity and NO production in HUVEC

HUVEC, normally expressing eNOS in membrane fractions, were first treated with ESW at a potency of 0.03 mJ/mm^2 and thereafter, the membrane-associated enzyme activity was estimated. ESW rapidly increased eNOS activity in HUVEC, this increase being dependent on the number of ESW shots (500–1500), with a maximum peak at 1000 shots (Fig. 1A).

Once having assessed that ESW enhanced eNOS activity in HUVEC, we measured NO intracellular production using confocal microscopy and a specific NO fluorescent probe (DAF2-DA). While in control cells diffused fluorescence was observed in cytoplasm, ESW treatment of HUVEC with 1000 shots at the energy level of 0.03 mJ/mm^2 significantly enhanced the overall fluorescence. This increase was significantly reduced when cells were pre-incubated for 2 h with 1 mM *N*-nitro-L-arginine methyl ester (L-NAME), a strong NOS inhibitor (Figs. 1B and C).

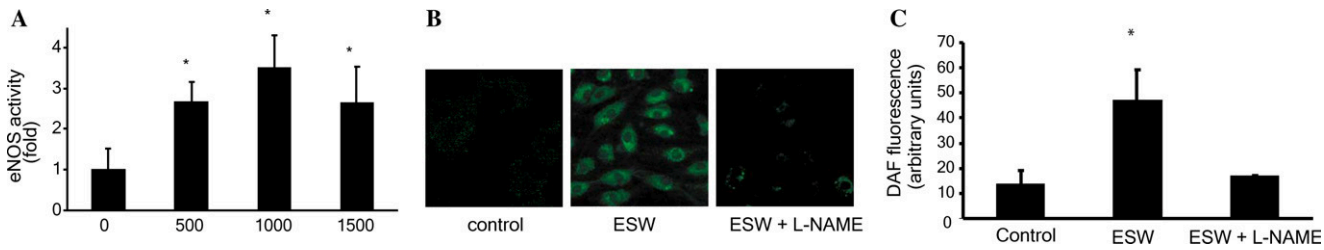

Fig. 1. Modulation of eNOS activity and NO production by ESW treatment. (A) HUVEC were treated at the energy level of 0.03 mJ/mm^{2}with a different number of ESW shots and eNOS activity was measured. Data are shown as fold increase (means ± SD, $n = 6$); *$P < 0.005$ versus not treated cells. (B) Confocal images of HUVEC treated with ESW in the presence and absence of 1 mM L-NAME. Control represents non-treated HUVEC. Magnification: 40×. (C) DAF-2T fluorescence of HUVEC treated with ESW in the presence and in the absence of 1 mM L-NAME. DAF fluorescence is expressed as arbitrary units; digital scale ranging from 0 to 255, minimum to maximum fluorescence intensity, respectively. The mean fluorescence was calculated from 20 to 40 cells/observation field; two observations field/treatment condition/experiment. *$P < 0.005$ versus not treated cells.

Effect of ESW on eNOS phosphorylation state in HUVEC

To verify eNOS phosphorylation state, HUVEC were first treated with different inhibitors of the phosphorylation pathway. eNOS activity slightly increased when cells were treated with 1 mM genistein, a potent tyrosine kinase inhibitor, while it decreased to 60% of the basal value in the presence of 1 mM sodium orthovanadate, a potent tyrosine phosphatase inhibitor (Fig. 2A). When cells were treated with 25 μM LY294002 and 200 μM wortmannin, two inhibitors of PI3K, eNOS activity was found to have decreased (to 40 and 60% of the basal value, respectively). One hundred nanomolar of okadaic acid, a protein phosphatase 1 and protein phosphatase 2A inhibitor, had an opposite effect (Fig. 2A). These data were further confirmed by measuring NO production in HUVEC with confocal microscopy using DAF2-DA as NO probe (data not shown).

Once having ascertained that eNOS activity in HUVEC is modulated by serine- and tyrosine-phosphorylation (Fig. 2B), we investigated on whether ESW mechanism of action may involve modulation of the enzyme phosphorylation state. As shown in Fig. 2A, ESW-elicited enhancement of eNOS activity was reverted only when cells were incubated with sodium orthovanadate.

Effect of ESW on eNOS activity in LPS/IFN-γ-treated HUVEC

We used HUVEC stressed with lipopolysaccharides (LPS)/interferon-γ (IFN-γ) as an in vitro acute inflammation model. HUVEC were treated with 1 μg/ml LPS plus 10 ng/ml IFN-γ for 2 h and eNOS activity and NO production were assessed thereafter. As expected, eNOS activity had dropped to 30% of the basal value (Fig. 3A). In accordance with these results, the experiments performed using DAF-2DA showed a significantly less fluorescence in LPS/IFN-γ-treated HUVEC than that in control cells, indicating that LPS/IFN-γ-treatment indeed caused intracellular NO production to sharply drop (Figs. 3B and C). ESW, 1000 shots at 0.03 mJ/mm^2, counteracted the inhibitory action of LPS/IFN-γ on eNOS activity (Fig. 3A) and on NO production (Fig. 3B) keeping them at the basal value.

Effect of ESW on NF-κB activation in LPS/IFN-γ-treated HUVEC

LPS/IFN-γ elicited a rapid activation of NF-κB in HUVEC (Fig. 4). To ascertain whether ESW can possibly suppress NF-κB activation by up-regulating eNOS

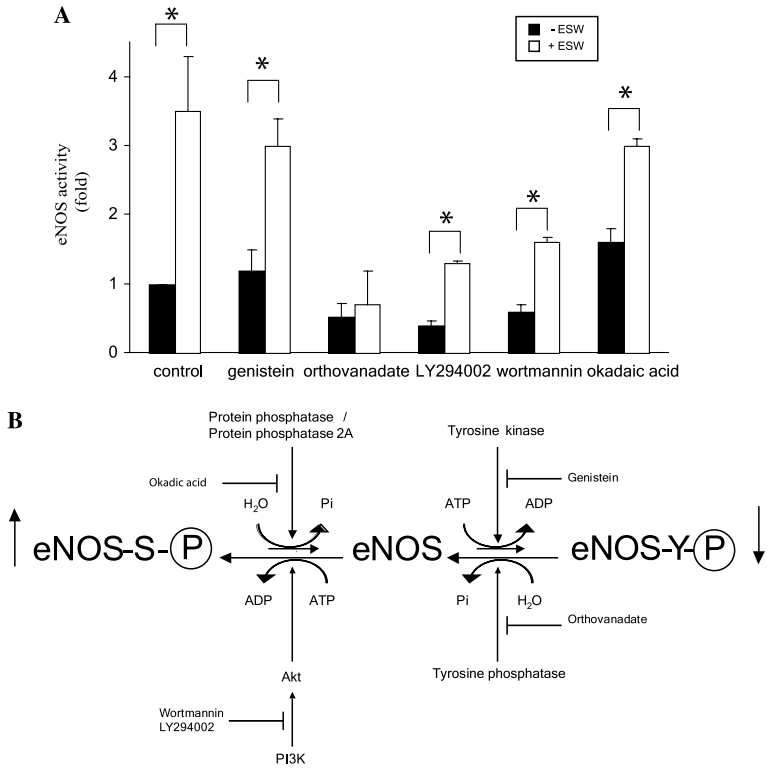

Fig. 2. Modulation of eNOS phosphorylation state by ESW treatment. (A) HUVEC were incubated for 2 h with 1 mM genistein or 1 mM sodium orthovanadate, for 30 min with 25 μM LY294002 or 200 μM wortmannin or 100 nM okadaic acid. Thereafter, cells, in the presence and absence of inhibitors, were treated with ESW (0.03 mJ/mm^2, 1000 shots) and eNOS activity was measured. Data are shown as fold increase (means ± SD, $n = 6$); *$P < 0.05$. (B) Phosphorylation state of eNOS in HUVEC. eNOS-S-P represents serine-phosphorylated eNOS and is more active than the native form; eNOS-Y-P represents tyrosine-phosphorylated eNOS and is less active than the native one. Symbols ↑ and ↓ represent, respectively, more and less active form of eNOS compared to native enzyme.

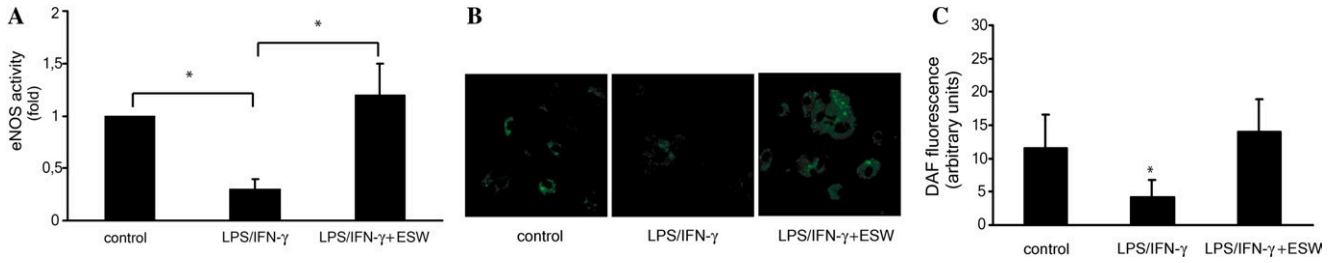

Fig. 3. Effect of ESW on eNOS activity in LPS/IFN-γ-treated HUVEC. (A) HUVEC incubated for 2 h with LPS/IFN-γ were treated with ESW (0.03 mJ/mm^2, 1000 shots) and eNOS activity was measured. Data are shown as fold increase (means ± SD, $n = 10$); *$P < 0.05$. (B) Confocal images of HUVEC treated with ESW in the presence and in the absence of LPS/IFN-γ. Controls represent non-treated HUVEC. Magnification: 40×. (C) DAF-2T fluorescence of HUVEC treated with ESW in the presence and absence of LPS/IFN-γ. DAF fluorescence is expressed as arbitrary units; digital scale ranging from 0 to 255, minimum to maximum fluorescence intensity, respectively. The mean fluorescence was calculated from 20 to 40 cells/observation field; two observation field/treatment condition/experiment. *$P < 0.05$ versus not treated cells.

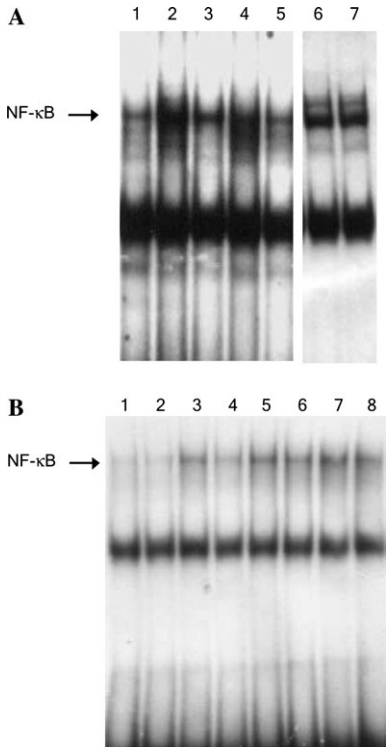

Fig. 4. Effect of ESW on LPS/IFN-γ-elicited NF-κB activation in HUVEC. (A) Lane 1, control cells; lanes 2 and 4, cells treated with LPS/IFN-γ; lane 3, cells treated with LPS/IFN-γ and ESW; lane 5, cells treated first with ESW and soon after with LPS/IFN-γ; lane 6, cells pre-incubated with 1 mM L-NAME for 2 h and then treated with LPS/IFN-γ; and lane 7, cells pre-incubated with 1 mM L-NAME for 2 h and then treated with LPS/IFN-γ and ESW. (B) Lane 1, control cells; lane 2, cells treated with ESW; lanes 3, 5, and 7, cells treated with LPS/IFN-γ for 30 min, 1 and 2 h, respectively; and lanes 4, 6, and 8, cells pre-incubated with LPS/IFN-γ for 30 min, 1 and 2 h, respectively, and then treated with ESW. In all EMSA experiments, cells were treated with 1000 shots of ESW at energy level of 0.03 mJ/mm^2.

activity, HUVEC were first treated with ESW (1000 shots at 0.03 mJ/mm^2) at the same time of, or just before LPS/IFN-γ administration to cell culture. After keeping the cells into the incubator for 30 min, DNA-binding activity of NF-κB was measured. Under these conditions ESW strongly inhibited LPS/IFN-γ-elicited activation of NF-κB (Fig. 4A). One millimolar of L-NAME, administrated to cell culture 2 h before ESW treatment, reverted completely the inhibitory effect of ESW on LPS/IFN-γ-elicited NF-κB activation (Fig. 4A).

To determine whether the effect of ESW treatment on NF-κB was observed also when NF-κB is already activated by LPS/IFN-γ, HUVEC were treated with ESW (1000 shots at 0.03 mJ/mm^2) at 30 min, 1, and 2 h after incubation with LPS/IFN-γ. ESW treatment reduced significantly DNA-binding activity of NF-κB at any time point, although the maximal effect was observed after 30 min (Fig. 4B).

Discussion

The present study attempts to identify the molecular mechanism of anti-inflammatory action of ESW which, in the last decade, has been successfully employed in the clinical treatment of different orthopaedic disorders. Clinical experiences, developed using this technique, showed the possibility to obtain an immediate anti-inflammatory result in soft tissue inflammation. Furthermore, experimental animal studies showed the neoangiogenetic role of this therapy in degenerative soft tissue diseases.

Although the biochemical mechanisms underlying these effects have to be elucidated yet, clinical observations of blood flow increase around treated area and successive enhancement in angiogenesis indicate that ESW-elicited anti-inflammatory action should be a NO-dependent phenomena.

In the attempt to identify if ESW may modulate NO production, we examined eNOS activity and intracellular accumulation of NO in normal and LPS/IFN-γ-treated HUVEC.

As shown in Fig. 1A, ESW, at the energy density corresponding to that employed in the clinical treatment of soft tissue inflammation (0.03 mJ/mm^2), rapidly enhanced eNOS catalytic activity in HUVEC. This increase was dependent on the number of ESW shots,

with a maximum peak at 1000 shots. In accordance with these results, ESW induced an NO intracellular accumulation (Figs. 1B and C) which was significantly reduced when cells were pre-incubated with L-NAME, a strong NOS inhibitor. eNOS activity in HUVEC is modulated by post-translational modification such as tyrosine- and serine-phosphorylation, indicating that overall NO production is regulated by the equilibrium between the enzyme different phosphorylated states. Results shown in Fig. 2 strongly indicate that ESW quickly increase eNOS activity by shifting the balance to a less-tyrosine-phosphorylated form. This notion arises further considerations. In line with results shown in Fig. 2, in human astrocytoma cells T67, eNOS was found to be normally tyrosine-phosphorylated [39] and in bovine aorta endothelial cells (BAEC) tyrosine-phosphorylation of eNOS was reported to down-regulate the enzyme activity [21]. However, some reports point out the possibility that tyrosine-phosphorylation pathway differently regulates eNOS activity. For instance, activation of eNOS in HUVEC after treatment with protein phosphatase inhibitor, phenylarsine oxide, was associated with tyrosine-dephosphorylation of the enzyme [28]. In BAEC, sodium orthovanadate stimulated bradykinin-mediated NO production due to changes in protein association [30]. Furthermore, sodium orthovanadate enhanced NO production in bovine lung microvascular cells (BLMVEC) by up-regulating Akt kinase leading to eNOS activation [40]. Therefore, despite the well-documented role of serine/threonine-phosphorylation on eNOS activity, modulation of eNOS activity by tyrosine-phosphorylation remains to be a matter of debate. In any case, data presented in Figs. 1 and 2 fit well with the clinically observed increase in the blood flow immediately following ESW treatment which could be due, at least partly, to the rapid increase in NO production around the ESW-treated site.

Next, we wanted to find out if ESW-elicited increase in NO production may account for a clinically observed anti-inflammatory action of this technique. To verify this hypothesis, we used HUVEC stressed with LPS/IFN-γ as an in vitro acute inflammation model which trigger NF-κB activation and successive expression of genes playing a critical role in inflammation.

As shown in Fig. 3, LPS/IFN-γ treatment induced a consistent decrease in eNOS activity and NO production. Again, ESW counteracted this effect, keeping NO concentration at the basal value, if not sometimes slightly over. Since, according to the previous evidences, the drop in NO and successive NF-κB activation might be a hallmark of the early phase of inflammatory event, we proposed that any treatment counteracting the decrease in the amounts of NO should modulate not only NF-κB activation but also successive whole inflammatory events [41]. As previously reported, LPS/IFN-γ induced a rapid time-dependent activation of NF-κB in HUVEC (Fig. 4B). ESW treatment at the same time or just before LPS/IFN-γ administration to the cell culture reverted this effect. All these data strongly indicate that the clinically observed anti-inflammatory action of ESW implies rapid up-regulation of eNOS activity and successive increase in NO output that may keep NF-κB activation suppressed at the early phase of inflammation, despite the presence of immune cytokines. Failure of ESW treatment in counteracting LPS/IFN-γ-induced inhibition of eNOS activity and NF-κB activation in the presence of L-NAME further strengthens this notion.

Furthermore, we present data indicating that ESW treatment down-regulates NF-κB not only before but also after its activation. Since most of patients treated with ESW owe for ongoing inflammatory events, the finding that ESW treatment is capable not only to prevent but also to down-regulate NF-κB activation seems to match better with clinical observations on an anti-inflammatory action of ESW treatment.

According to evidence in the literature, eNOS activity is modulated not only by the enzyme phosphorylation but also by the interaction with other proteins such as

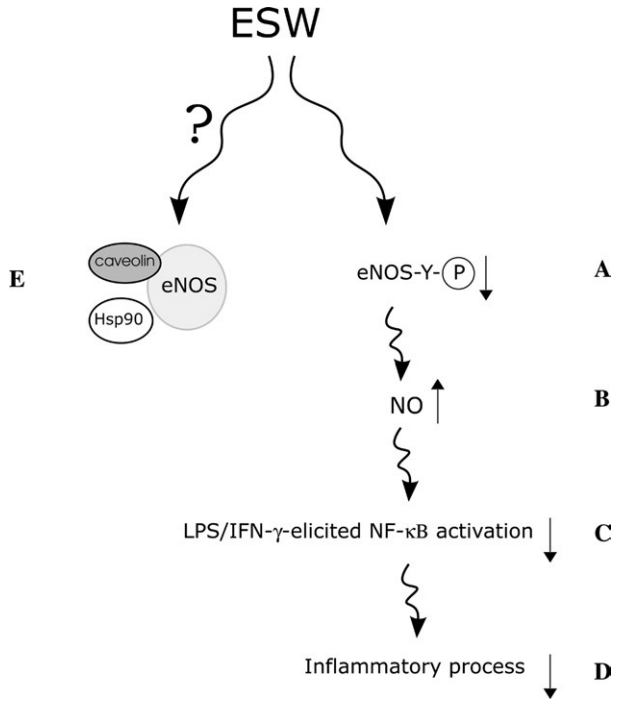

Fig. 5. General view on the molecular pathway involved in ESW-elicited anti-inflammatory effect. Experimental data described in the present study indicate the following pathway, leading to the down-regulation of inflammatory process elicited by ESW. ESW quickly induce tyrosine-dephosphorylation of eNOS (A), enhancing the enzyme catalytic activity. Successive increase in the amounts of NO (B) keep suppressed NF-κB activation despite the presence of LPS/IFN-γ (C). Lack of NF-κB activation inhibits LPS/IFN-γ-elicited expression of genes involved in the inflammatory process, down-modulating whole inflammatory process (D). Possible effect of ESW on association between eNOS and other proteins, such as caveolin and Hsp90, remains to be elucidated (E).

calmodulin [20], caveolin 1–3 [21], and Hsp90 [22]. Furthermore, a recent report describes a possibility that physical forces, such as shear stress, may induce a drastic change in cytoscheletal structure of endothelial cells [31] as well as an enhancement of eNOS activity by phosphorylation [28,32]. Since ESW at low potencies may elicit a similar force as that produced by shear stress [42], the possibility that ESW treatment may induce some changes in the interaction of eNOS with other proteins triggering the activation of eNOS activity, still stands as a fascinating working hypothesis.

In conclusion, our results show that ESW treatment quickly enhanced eNOS activity and intracellular NO production in HUVEC. These phenomena seem to be obtained by affecting tyrosine-phosphorylation of eNOS, i.e., by shifting the balance between the native and phosphorylated states to a less-tyrosine-phosphorylated one. LPS/IFN-γ-elicited NF-κB activation was efficiently inhibited by ESW treatment due to its action in keeping eNOS activity at the basal value. NO produced by eNOS should suppress NF-κB activation and down-regulate the inflammatory event (Fig. 5). The clinically observed anti-inflammatory action of ESW treatment, therefore, should be sustained, at least partly, by its quick action on eNOS activity.

Acknowledgments

This work was financially supported by FIRB Project 2001 and CIRC. We thank Marzia Di Chio for technical support and Luisella Mattace for editorial assistance.

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