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ACE-inhibition ameliorates vascular reactivity and delays diabetes outcome in NOD mice

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ABSTRACT

Recently, we have demonstrated a direct correlation among hyperglycaemia, vascular dysfunction and eNOS post-translational regulation in non non-obese diabetic mice (NOD). Here, we evaluate the impact of two ACE-inhibitors therapy, zofenopril and enalapril in NOD mice. Insulin-dependent diabetes mellitus (IDDM) development was monitored weekly through glycosuria measurement. Zofenopril and enalapril were dosed at 0.5 mg/kg/die orally. Animals were sacrificed at different points and aortas used for western blotting or for tissue bath experiments. Bovine aortic endothelial cells in high glucose medium are treated with zofenoprilat or enalaprilat. Cells and supernatant were utilised for western blot analysis and for nitrite/nitrate determination, respectively. In ex-vivo experiments chronic administration of both drugs restored PE-induced contraction but not Isop-induced vasodilatation, however only zofenopril reduced caveolin-1 expression. In vitro, both drugs inhibited caveolin-1 expression and increased NOx production. However, zofenopril caused inhibition of both parameters at a concentration 200 fold lower than enalalpril. In vivo, zofenopril delays the onset of diabetic conditions of about 50%, and ameliorates polyuria. In conclusion our data suggest that ACE-inhibitor therapy may be useful in IDDM, in particular sulphydrylated inhibitor would display a better efficacy especially if administered early on the development of diabetes.

1. Introduction

Type 1 diabetes, or insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease characterised by a islet inflammation or insulitis, followed by progressive destruction of pancreatic β cells and by insulin secretion deficiency, resulting in hyperglycaemia (Atkinson and MacLaren, 1994; Expert Comm. on diagnosis on diabetes mellitus, 1997). IDDM is considered one of the major risk factors for the development of cardiovascular pathologies, indeed the main cause of morbidity and premature mortality associated with IDDM (The diabetes control res. group, 1993) includes vascular dysfunctions such as atherosclerosis, macro- and microangiopathy, diabetic cardiomyopathy and nephropathy that converge in arterial hypertension (Keen et al., 1999; Oomen et al., 1999; Jensen-Urstad et al., 1996; Kennon et al., 1999). Consequently, prevention and management of endothelial dysfunction in diabetic patients is currently considered an important target for pharmacological intervention in IDDM-associated complicances (Buikema et al., 2000). In this context Angiotensin Converting Enzyme (ACE) inhibitors are widely used as therapeutics, and their ability in controlling hypertension and risk of cardiovascular death is well established both in experimental investigations and in clinical trials. However, it has been shown that ACE inhibitors, beyond their classical action on renin–angiotensin system, exert a protective action on cardiovascular system. In fact they improve endothelial function, cardiac and vascular remodelling and they have been shown to reduce atherogenesis in experimental models with different mechanisms (Hayek et al., 1998; Hayek et al., 1999; Keidar et al., 2000; de Nigris et al., 2001; Chobanian et al., 1990; Rolland et al., 1991; Kowala et al., 1994) such as antiproliferative effects on vascular smooth muscle cells (Daemen et al., 1991; Li et al., 1999), reduction of low-density lipoprotein (LDL) oxidation (de Nigris et al., 2001; Napoli et al., 1999; Keidar et al., 2000), modulation of proinflammatory signals in the vasculature (Gonzales et al., 2000) and improvement of endothelial disorders (Buikema et al., 2000, Rolland et al., 1991).

Recently, our group has demonstrated a direct correlation between hyperglycaemia and vascular dysfunction in non non-obese diabetic mice (NOD). In this strain progressive development of diabetic condition matches with an increasing vascular hypo-functionality (Bucci et al., 2004). The purpose of this paper was to evaluate the impact of ACE-inhibitor therapy in NOD mice by using two ACE-inhibitors namely enalapril and zofenopril.

2. Methods

2.1. Experimental design

Female Non Obese Diabetic mice (NOD/Ltj) and CD-1 mice, were purchased from Charles River (Italy). NOD mice exhibit a susceptibility

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Table 1
Glycaemia and glycosuria values in the three different groups of NOD mice (NOD groups I, II and III respectively) related to the age

	NOD I	NOD II	NOD III
Glycaemia (mg/dl)	100±12	220±15	490±25
Glycosuria (mg/dl)	5±1	65±18	1000±100
Age (weeks)	5±2	13±3	22±3

Glycaemia normal value: 110±20 mg/dl.

to spontaneous development of autoimmune (type I) insulin insulin-dependent diabetes mellitus (IDDM) (Makino et al., 1980). The study was performed with female mice because they have much higher incidence of developing diabetes (Gross et al., 2008; Sreenan et al., 1999; Han et al., 2008; Choi et al., 2008). Diabetes development in NOD mice is characterised by insulitis and leukocytic infiltrate of the pancreatic islets. Progressive reduction in pancreatic insulin content starts at about 12–16 weeks of age (Table 1). In order to evaluate IDDM development in these animals a measurement of glycosuria was performed weekly starting from 5 weeks of age. Mice are divided in the following three groups:

- NOD group *I* 0<gl<20 mg/dl = low or null glycosuria
- NOD group *II* 20<gl<500 mg/dl = high glycosuria
- NOD group *III* 500<gl to 1000 mg/dl = severe glycosuria.

This classification allows to differentiate the treatments in preventive or therapeutic regimens. To perform the preventive regimen mice belonging to group I were randomised to receive either zofenopril (Zf) or enalapril (En) starting at 5 weeks of age. Conversely in order to evaluate the effect of Zf and En in a therapeutic approach (e.g. when glycaemia is already at pathological concentration) we randomised mice that had reached glycosuria levels to group II or III to be treated with either Zf or En, respectively 20–500 mg/dl for group II (13±3 weeks of age) and 500–1000 mg/dl for group III (22±3 week s of age) (for details see Bucci et al., 2004). Both drugs were dosed at 0.5 mg/kg/die since this dose is strictly related to doses used in therapy (Borghi and Cicero, 2006; Pasini et al., 2007; Borghi et al., 2007). During all treatments the glycosuria was monitored weekly and mice were sacrificed when the value of glycosuria of a specific group reached the NOD *III* stage. Drugs are dissolved in carboxy methyl cellulose 0.2% w/v in saline. Drugs or vehicle were was administrated per oral gavage in a volume of 200 µl.

2.2. Measurement of glycosuria

To assess the diabetic condition of NOD animals, glycosuria was evaluated weekly to select the animals. This method was used since it is non non-invasive and it well correlates with an increase in blood glucose (Bucci et al., 2004). Briefly, groups of 3 mice were placed in metabolic cages able to selectively collect urine, for at least 4 h. The content of glucose in the urine was measured by using Trinder reaction (Glucose Trinder 100, Sigma Chemical Co. Milano, Italy). This method is based on the glucose oxidation to gluconic acid and hydrogen peroxide in the reaction catalysed by glucose oxidase (Trinder, 1969). The value of absorbance (λ=505 nm) is directly proportional to the glucose concentration in the sample. At these different points, blood sugar levels were determined before the animals were sacrificed. Aortas were dissected and used for western blotting analysis or for tissue bath experiments. Experiments were performed also on CD-1 mice in order to have an internal non non-diabetic control to compare with NOD I. In addition, the volume of urine collected was measured in order to evaluate if zofenopril and enalapril treatments could influence the polyuria (increase of urine volume) typical of diabetic conditions.

2.3. Tissue preparation

NOD or CD-1 mice were sacrificed and thoracic aorta was rapidly dissected and cleaned from fat and connective tissue. Rings of 1.5–2 mm length were cut and mounted on wire myographs (Kent Instruments, Japan) filled with gassed Krebs solution (95% O_2+5% CO_2) at 37 °C. Changes in isometric tension were recorded with PowerLab data acquisition system (Ugo Basile, Italy).The composition of the Krebs solution was as follows (mol/l): NaCl 0.118, KCl 0.0047, $MgCl_2$ 0.0012, KH_2PO_4 0.0012, $CaCl_2$ 0.0025, $NaHCO_3$ 0.025 and glucose 0.010. Rings were initially stretched until a resting tension of 1.5 g was reached and allowed to equilibrate for at least 40 min during which tension was adjusted, when necessary, to 1.5 g and bathing solution was periodically changed. In a preliminary study a resting tension of 1.5 g was found to develop the optimal tension to stimulation with contracting agents.

2.4. Tissue experimental protocols

In each experiment rings were firstly challenged with L-phenylephrine (PE) 1 µmol/l until the responses were reproducible. When the tissue was unresponsive to PE (i.e. in NOD III) the first challenge was performed with serotonin (5-HT) 1 µmol/l. To evaluate tissue contractility, cumulative concentration response curve to PE (10 nmol/l–30 µmol/l) was performed. Conversely, to evaluate tissue vasorelaxation, cumulative concentration response curve to isoproterenol (Isop) (10 nmol/l–30 µmol/l) were was performed on PE-precontracted rings.

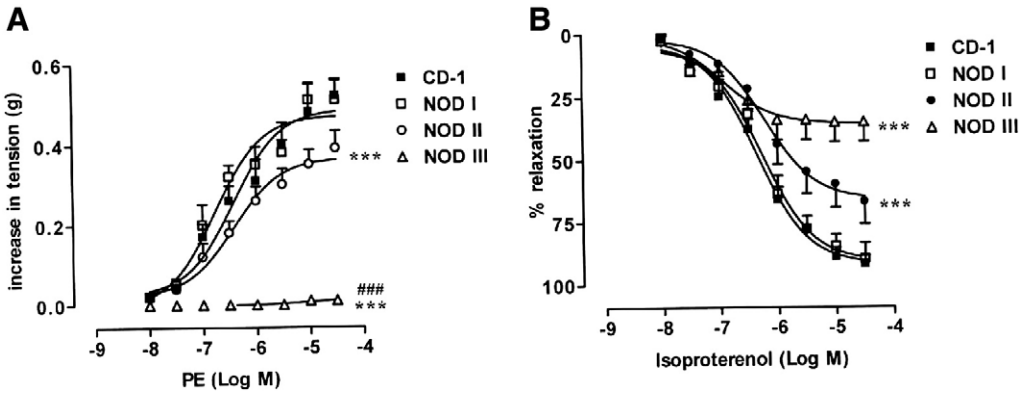

Fig. 1. (A) Effect of PE on aortic rings harvested from NOD groups I, II, III and CD-1 mice. PE-induced cumulative concentration response curve (10 nM–30 µM) was reduced in NOD group II (***p<0.001) compared to NOD group I and CD-1 mice. In NOD group III PE-induced vasoconstriction is strongly inhibited (***p<0.001 vs. NOD group I and CD-1 mice; ### p<0.001 vs. NOD group II). Data are presented as mean±S.E.M. of tension increase, n=8 for each group. (B) Evaluation of vasorelaxant effect of Isop on aortic rings harvested from NOD groups I, II, III and CD-1 mice. Isop-induced cumulative concentration response curve (10 nM–30 µM) was progressively reduced in NOD groups II and III compared to NOD group I and CD-1 mice. In particular, Isop-induced vasodilatation in NOD group II was significantly diminished when compared to NOD group I and CD-1 mice (***p<0.001). Reduced vasodilatation was even more pronounced in NOD group III mice (***p<0.001 vs. NOD group I and CD-1 mice; ## p<0.01 vs. NOD group II). Data are presented as mean±SE of % of vasodilatation, n=8 for each group.

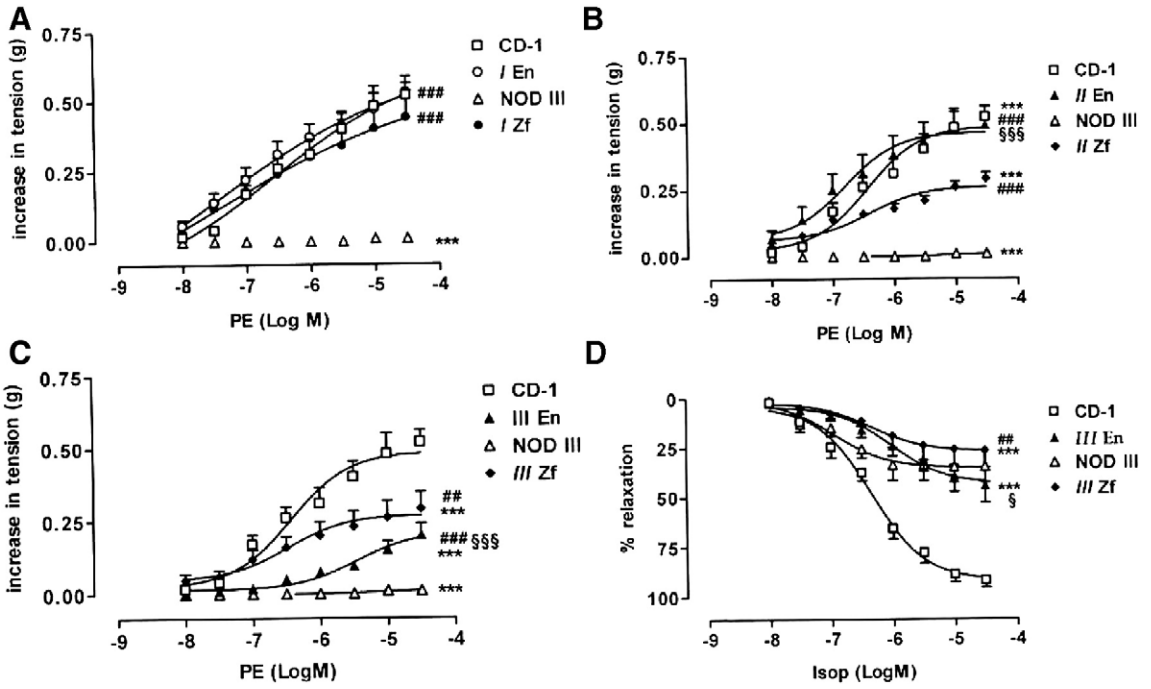

Fig. 2. Effect of chronic administration of zofenopril and enalapril on adrenergic receptors-induced vascular reactivity. After standardization procedure a PE-induced cumulative concentration response curve (A, B, C) or Isop-induced cumulative concentration response curve (D) were was performed (10^{-8}–3×10^{-5} M). PE-induced cumulative concentration response curve was performed in mice from group NOD I (A), group NOD II (B), and group NOD III (C); Isop-induced cumulative concentration response curve was performed in mice from group NOD III (D). Data are presented as mean±S.E.M. ***$p<0.001$ vs. CD-1; ### $p<0.001$ vs. NOD III; §§§ $p<0.001$ vs. Zf; $n=6$ for each group of mice.

2.5. BAEC with normal and high glucose

Bovine aortic endothelial cells (BAEC) cells were obtained by Istituto Nazionale per lo Studio e la Cura dei Tumori (Milano, Italy). The cells were cultured in 60 mm Petri plastic dishes (FALCON, Microtech Italy) and grown in medium (GIBCO, Invitrogen Corporation) supplemented with 2 mmol/l glutamine (GIBCO), 10% heat inactivated fetal calf serum (GIBCO), 50 U/ml penicillin, and 50 U/ml streptomycin. The Petri dishes were incubated at 37 °C in a 5%CO_2–95% air gas mixture. BAEC were subcultured on reaching confluence by the use of 0.01% trypsin–EDTA. The cells were used between passages 5 and 6. BAEC were grown until they reached 90% confluence and then were serum starved overnight. Then cells were incubated for 3 h with medium containing 11.5 mM D-glucose (normal glucose) or 25 mM D-glucose (high glucose) (Kimura et al., 2001). After 3 h pre-treatment with glucose, BAEC were incubated with zofenoprilat or enalaprilat (water soluble salts of zofenopril and enalapril respectively) at concentration of 0.3, 1, 10 and 60 μM or vehicle for other 3 h. Immediately thereafter cells were stimulated for 30 min with calcium ionophore A23187 (10 μmol/l) (Bucci et al., 2004). Cells were then separated from the

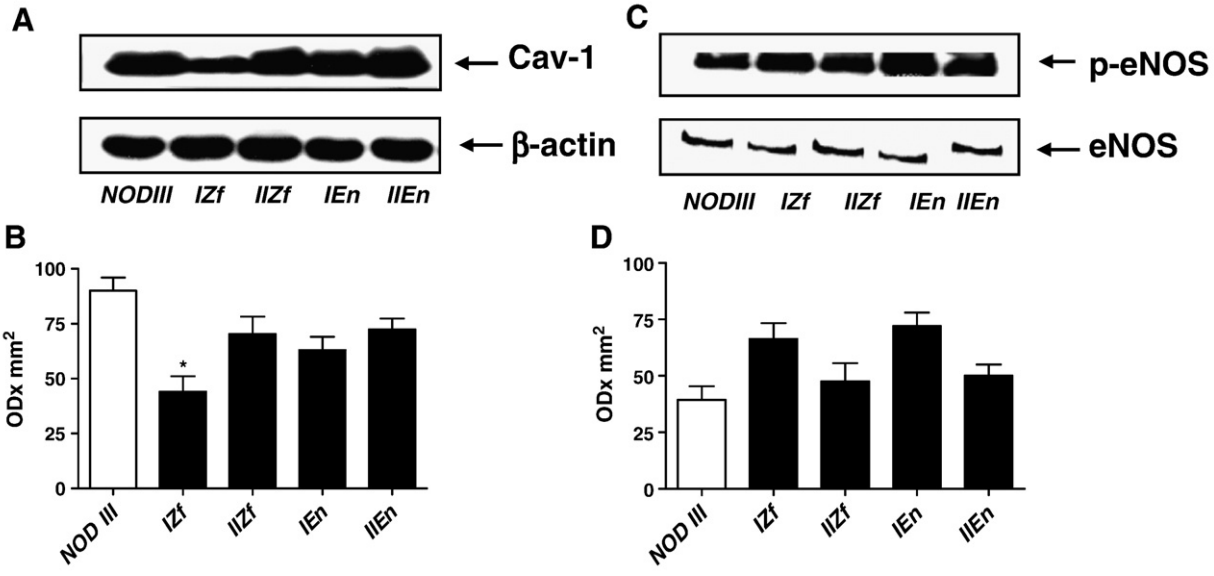

Fig. 3. Effect of chronic administration of zofenopril and Enalapril on eNOS, phospho-eNOS and Cav-1 expression in NOD mice subjected to different regimens of administration. Aortic tissues were harvested from NOD III, NOD groups I and II of zofenopril (IZf and IIZf respectively), and NOD groups I and II of Enalapril (IEn and IIEn respectively). (A) The figure is representative western blotting from three independent experiments. (B) Densitometric analysis of caveolin-1 blots. *$p<0.05$ vs. NOD III. (C) The figure is representative western blotting from three independent experiments. (D) Densitometric analysis of phospho-eNOS/eNOS ratio.

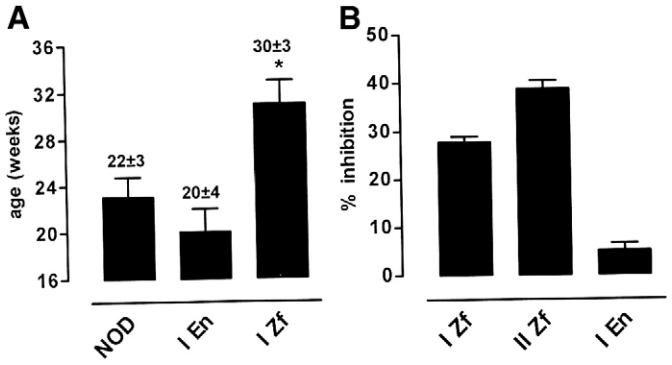

Fig. 4. (A) Effect of chronic treatment with zofenopril and enalapril of NOD groups I (IEn and IZf), compared to NOD mice, on the onset of diabetic conditions. Numbers above the bars represent time in weeks, necessary to get NOD group III glycosuria levels. To assess the diabetic condition of NOD animals, glycosuria was evaluated weekly to select the animals. Briefly, groups of 3 mice were placed in metabolic cages able to selectively collect urine, for at least 4 h. Data are express as mean ± S.E.M each group $n = 5$, $*p < 0.05$ vs. unNOD. (B) Effect of chronic treatment with zofenopril and enalapril of NOD groups I and II (IZf, IIZf and IEn) on polyuria. The treatments with Zf inhibited the amount of urine produced over 30%. To evaluate the amount of urine produced groups of 3 mice were placed in metabolic cages able to selectively collect urine for 4 h. The urine volume was measured by using graduated tubes. The control value of urine production is 278 ± 16.7 µl/mouse.

supernatant and utilised for western blot analysis while the medium was used for nitrite/nitrate (NOx) determination.

2.6. NOx determination

Cellular supernatant and a standard curve of sodium nitrate were incubated in a microplate with cadmium (50 mg/well) for 1 h to convert NO_3^- to NO_2^- (Thomsen et al., 1990). After centrifugation at 14,000 rpm for 15 min, total nitrite (NOx) content was determined fluorometrically in microtiter plates using a standard curve of sodium nitrite (Misko et al., 1993). NOx content was calculated by using the internal standard curve.

2.7. Western blotting

Aortic tissue samples or BAEC were homogenised in lysis buffer (β-glycerophosphate 0.5 M, sodium orthovanadate 10 mM, $MgCl_2$ 20 mM, EGTA 10 mM, DTT 100 mM and protease inhibitors) using a Talon homogenizer, and were processed identically. Protein concentration was determined using Bradford assay (Bio-Rad Laboratories, Segrate, MI). Proteins (30 µg) were subjected to electrophoresis on an SDS 10% polyacrylamide gel and electrophoretically transferred onto a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Germany). The immunoblots were developed with 1:1000 dilutions for eNOS, p-eNOS and caveolin-1, and the signal was detected with the ECL System according to the manufacturer's instructions (Amersham Pharmacia Biotech).

2.8. Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was performed by using 2-way or 1-way ANOVA followed by a multiple comparison test where appropriate. Differences were considered statistically significant when P value was less than 0.05.

2.9. Reagents

L-phenylephrine (PE), serotonin (5-HT), and isoproterenol (Isop) were purchased from Sigma Chemical Co. (Milano-Italy). All salts used

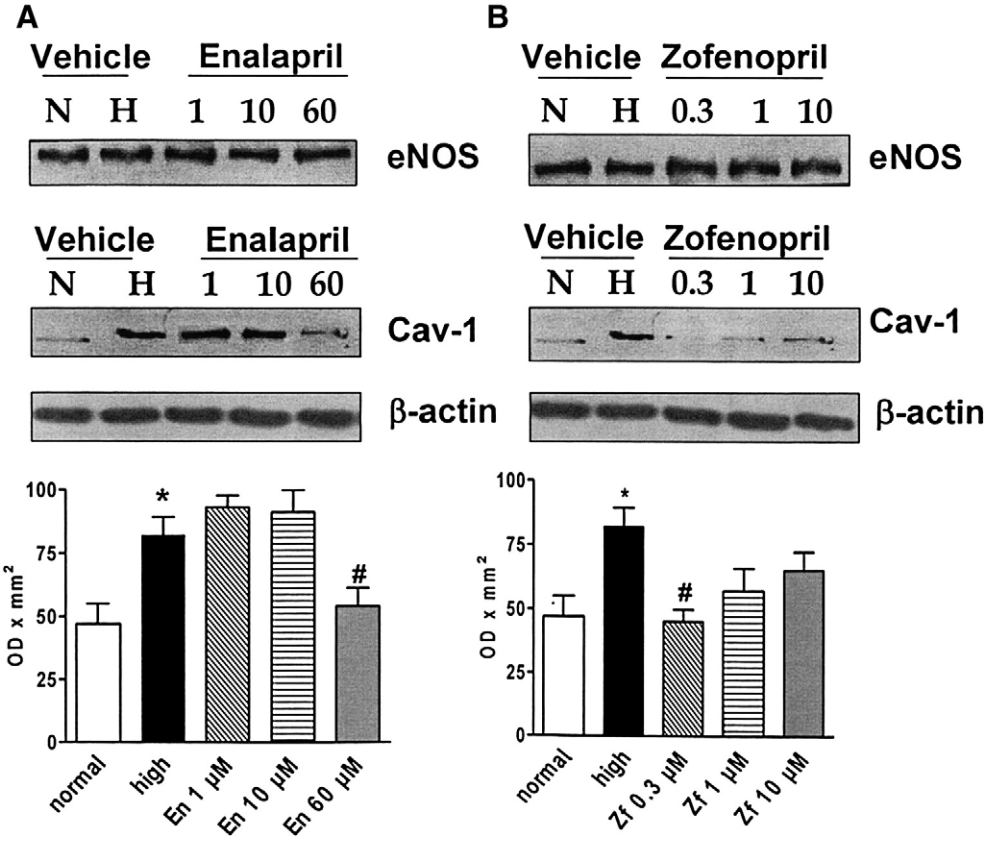

Fig. 5. Effect of enalapril (A) and zofenopril (B) on eNOS and caveolin expression in BAEC exposed to high glucose. After 3 h pre-treatment with glucose, BAEC were incubated with zofenoprilat or enalaprilat (water soluble salts of zofenopril and enalapril respectively) at concentration of 0.3, 1, 10 and 60 µM or vehicle for other 3 h. Immediately thereafter cells were stimulated for 30 min with calcium ionophore A23187 (10 µmol/l). Cells were then separated from the supernatant and utilised for western blot analysis. Western blots are representative of three separated experiments each. Relative densitometric analysis values are mean ± S.E.M. $*p < 0.05$ vs. normal glucose, $\#p < 0.05$ vs. high glucose.

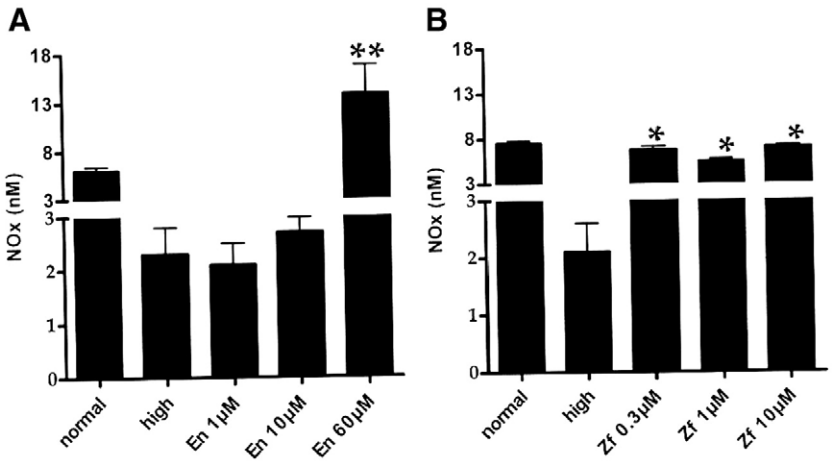

Fig. 6. Effect of zofenopril (B) and enalapril (A) on NOx production in BAEC exposed to high glucose. Cellular supernatant and a standard curve of sodium nitrate were incubated in a microplate with cadmium (50 mg/well) for 1 h to convert NO_3^- to NO_2^-. After centrifugation, total nitrite (NOx) content was determined fluorometrically in microtiter plates using a standard curve of sodium nitrite. NOx content was calculated by using the internal standard curve. Data are presented as mean ± S.E.M, $n = 3$, ** $p < 0.01$; * $p < 0.05$ vs. high glucose.

for Krebs solution preparation were purchased from Carlo Erba Reagenti (Milan, Italy). Anti-caveolin-1 IgG were was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California, USA). Anti-eNOS and anti p-eNOS were purchased from Calbiochem (EMD Chemicals, NJ USA). All salts used for western blot analysis were purchased from ICN Biochemical (Eschwege, Germany). Zofenopril, enalapril and their respective sodium salts zofenoprilat and enalaprilat were provided by Menarini Ricerche.

3. Results

3.1. Zofenopril and enalapril treatments restore PE-induced contraction but not Isop-induced vasodilatation in I, II and III groups

Aortic rings isolated from NOD mice, display an impaired PE-induced contraction (an α_1 adrenergic agonist) closely related to the disease progression (Bucci et al., 2004); indeed PE-induced contraction is abolished in NOD III mice (Fig. 1A). At the same time Isop-induced vasorelaxation (a β_2 adrenergic agonist) results strongly curtailed in NOD III mice (Fig. 1B). Chronic administration of both enalalpril and zofenopril in group I completely restored PE-induced vasoconstriction (Fig. 2A). In groups II (Fig. 2B) and III (Fig. 2C) instead, the treatments with these ACE-inhibitors only partially restored PE-induced vasoconstriction. Conversely, chronic administration of both enalapril and zofenopril did not influence Isop-induced vasodilatation in group III (Fig. 2D) as well as in groups I and II (data not shown). This experimental evidence suggests that both enalapril and zofenopril exert their beneficial effect on α_1 contracting tone when they are administrated during the onset of the diabetic condition. Such action become less marked, but still significant, if the treatment starts when the diabetes is already established.

3.2. Zofenopril treatment reduces caveolin-1 expression in group I

Our group has recently shown that the impaired production of endothelial-derived nitric oxide is not to be ascribed solely to a reduction of eNOS expression but to an increased expression of caveolin-1, a protein that negatively regulates eNOS activity (Fig. 3, Bucci et al., 2004). Zofenopril preventive treatment in group I restored the expression of caveolin-1 to physiological levels (Fig. 3B). Conversely, when zofenopril was administrated to group II and group III it did not affect caveolin-1 expression, suggesting that once the diabetic condition is in advanced state there is no therapeutic effect. In particular it should be noted that enalapril did not affect caveolin-1 expression (Fig. 3B). In order to evaluate if ACE-inhibitors treatment could influence eNOS activation at post-translational level, a western blot analysis on aortic tissues harvested from NOD group III mice treated with zofenopril, enalapril or vehicle for phospho-eNOS was performed. As shown in Fig. 3C both zofenopril and enalapril, in preventive regimen, increased eNOS phosphorylation compared to vehicle, the latter data are evaluated as densitometric ratio of phospho-eNOS/eNOS (Fig. 3D). This result is in line with the widely accepted concept that ACE inhibitors, beyond their classical action on renin–angiotensin system, exert a protective action on cardiovascular system improving endothelial function.

3.3. Zofenopril but not enalapril treatment delays the onset of diabetic conditions in group I

As we have recently demonstrated in NOD mice, measurement of glycosuria becomes predictive of diabetic pathology when the animals are 13–16 weeks of age. Progressive development of diabetic condition leads to an increase of the value of glycosuria in the range of NOD III in about 20–23 weeks of age (Bucci et al., 2004). Thus, animals reach NOD III condition within 20–22 weeks age. Mice belonging to group I received either Zf or En in a preventive fashion, e.g. when they still have normal glycaemia. When Zf was administrated to group I it significantly prolonged this period of time to 30 weeks of age (Fig. 4A). On the other hand if zofenopril was administrated to group II and group III, mice that already developed high or severe glycosuria respectively, there were no changes in diabetic outcome (data not shown). Enalapril treatment, instead did not have any effect on this parameter.

3.4. Zofenopril but not enalapril treatment ameliorates polyuria in groups I and II

A typical symptom of hyperglycaemic condition is represented by a significant polyuria. Daily treatment with Zf of group I and group II significantly reduced the polyuria (Fig. 4B). Zf did not have any effect on group III while En was inactive in the therapeutic and preventive protocols.

3.5. Zofenopril and enalapril reduce caveolin-1 expression and increase NOx production in BAEC with high glucose

Next, in order to investigate on molecular target for zofenopril and/or enalapril, we assessed if there was a modulation of eNOS post-translational activation. To address this specific issue we used BAEC. In basal conditions BAEC produce an amount of NO, measurable as total

nitrite, of about 2 nmol/l. When cells are stimulated with calcium ionophore, NO production increases up to 13 nmol/l (Bucci et al., 2004). In high glucose medium, BAEC produce the same amount of nitrite in basal conditions but, when cells are stimulated with calcium ionophore, a significant reduction of nitrite production occurs. Thus, high glucose environment negatively modulates eNOS activity (Bucci et al., 2004). Likewise to what happens in vivo, also in vitro the reduction of NO production is not to be ascribed to an inhibition of eNOS expression but to an increased expression of caveolin-1 (Bucci et al., 2004). When BAEC were treated in vitro with a concentration range mimicking, as much closer was possible, the plasma levels of zofenopril or enalapril generated by the chronic in vivo administration, both drugs inhibited caveolin-1 expression (Fig. 5A–B). However, zofenoprilat (sodium salt of zofenopril) exhibited this effect at a concentration 200 fold lower than enalalprilat (sodium salt of enalapril) that resulted active only at higher dose tested i.e. 60 μM. Similarly, both zofenoprilat and enalaprilat increased significantly NOx production (Fig. 6), also in this case zofenoprilat exerted its effect at a concentration much lower than enalaprilat suggesting a more potent and specific action.

4. Discussion

The notion that ACE-inhibitors therapy is one of the most effective in managing arterial hypertension is widely accepted. Indeed, it is known that ACE-inhibitor treatment slows the progression of renal disease (Lewis et al., 1993; Ravid et al., 1998; Kventy et al., 2001), and reduces cardiac failure in individuals with diabetes (Yusuf et al., 2000; Niskanen et al., 2001). However, the beneficial effect on vascular structure and activity remains to be elucidated. In our study we have utilised NOD mouse strain that spontaneously develops autoimmune type I diabetes with remarkable analogy to human IDDM (Makino et al., 1980; Kikutani and Makino, 1992) in order to evaluate the impact of two ACE-inhibitor therapy, zofenopril and enalapril, on vascular reactivity. In addition we monitored two different clinical parameters characteristics of diabetic conditions e.g. polyuria and glycosuria, that are also a feature of NOD mice. Aortas harvested from NOD mice display a progressive loss of α_1 adrenergic-induced contraction, coupled to a reduced expression of α_1 adrenergic receptor, that is closely related to the disease gravity (Bucci et al., 2004). When mice were treated before the development of the disease (e.g. group I) with either zofenopril or enalapril vessel contractility to phenylephrine was restored. This effect was not linked to a rescue of α_1 adrenergic receptor expression by zofenopril and enalapril treatments since there was no significant difference in its expression when compared to tissue harvested from NOD III mice. In other words the recovery of the responsiveness to PE was not linked to a rescue effect on the deletion of the adrenergic receptor. Since an increase in caveolin 1 expression impairs the adrenergic response (Je et al., 2004) and Cav-1 is also involved in eNOS regulation (Sessa, 2005) we have investigated on Cav-1 involvement. Interestingly, evaluation of Cav-1 expression on aorta harvested from mice treated preventively with zofenopril clearly showed that there was a decrease in Cav-1 expression that is a key event for PE-dependent contractions (Je et al., 2004). On the other hand, enalapril did not display the same effect suggesting that the mechanism underlying the effect on the adrenergic receptor was only one of the factor involved. As stated above Cav-1 is also a key regulator for eNOS activation. Since the endothelial dysfunction is the major cause of vascular impairment associated to the diabetes we used BAEC to address this specific matter. BAEC were exposed to a high or normal glucose environment in the presence of different concentration of either enalapril or zofenopril. Both zofenopril and enalapril reduced Cav-1 expression in a dose dependent manner. NOx production, that is impaired by the high glucose treatment, was also significantly prevented by both enalapril and zofenopril. However, zofenopril caused a reversion of the effect at a dose 200 fold lower than enalapril. Thus, most likely the difference in activity between zofenopril and enalapril found on Cav-1 expression is linked to a difference in potency rather than mechanistic. Zofenopril is one of the most efficacious drug among the ACE-inhibitors in ameliorating cardiovascular complications correlated to several pathologies. In particular, it has been shown that early treatment with zofenopril improves the clinical outcome of diabetic patients with myocardial infarction (Borghi and Ambrosioni, 2003a; Ambrosioni et al., 1995; Borghi et al., 2003b; Ambrosioni et al., 2001). This latter data fit with ours since, also in our experimental setting, the time frame of administration is crucial in order to reach a consistent beneficial effect. Concerning the positive action of ACE-inhibitor therapy on clinical signs of the diabetic pathology we observed that prophylactic regimen with zofenopril delayed the onset of the disease by significantly increasing the time window needed for the disease to develop naturally. Similarly, treatment of NOD group I with zofenopril, but not with enalapril, ameliorated polyuria, such beneficial effects on clinical signs of IDDM may be consequent of amelioration of vascular functionality. However, these effects are comforted by a striking literature that ascribes to ACE inhibitors a positive modulation on different parameters involved in the so called “cardiovascular inflammation response”. In fact, it has been shown that, in vitro, zofenopril inhibits the expression of adhesion molecules on endothelial cells by reducing reactive oxygen species (Cominacini et al., 2002) slowing the development of atherosclerosis. The difference in activity found could be ascribed to a sulfhydryl group present in zofenopril active metabolite (Subissi et al., 1999). Indeed, it is known that zofenopril is a pro-drug and its metabolite, namely zofenoprilat, is produced in vivo by cleavage of an esteric bond (Frascarelli et al., 2004). Zofenoprilat is highly lipophilic, more than any other ACE-inhibitor (Subissi et al., 1999), that ensures effective long-lasting tissue penetration (Sun and Mendelsohn, 1991; Evangelista et al., 2002; Evangelista and Mansini, 2005), uptake and the extent and duration of the ACE-inhibition at cardiovascular level (Subissi et al., 1999; Cushman et al., 1989a,b). In addition the amount of zofenoprilat bound to circulating ACE, makes its SH-groups to extracellular compartment in the vascular wall available where it can stabilize NO through formation of R-SNO and/or prevent its breakdown by scavenging free radicals in the extra-cellular environment (Buikema et al., 2000).

In conclusion ACE-inhibitor therapy may turn to be useful in patients with type 1 diabetes and also suggest that sulphydrylated inhibitor may display a better effect as long it is administrated at earlier stage of diabetes, as already suggested in clinical studies for other therapeutic uses (Borghi and Ambrosioni, 2003a; Ambrosioni et al., 1995; Borghi et al., 2003b; Ambrosioni et al., 2001).

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