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Long-term treatment with the apolipoprotein mimetic peptide D-4F increases antioxidants and vascular repair in Type I diabetic rats

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Short title: Treatment with D-4F improves vascular repair

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Non-Abbreviations

CO: carbon monoxide

D-4F: apolipoprotein A1 mimetic peptide

DM: diabetes mellitus

EC: endothelial cells

eNOS: endothelial nitric oxide synthase

EPC: endothelial progenitor cells

HDL: high density lipoprotein

i.p.: intraperitoneal

LDL: low density lipoprotein

MI: myocardial infarction

NO: nitric oxide

O_2^-: superoxide anion

ROS: reactive oxygen species

SOD: superoxide dismutase

TM: thrombomodulin

HO: heme oxygenase

CD_{31}: endothelial cell marker

STZ: streptozotocin

GSH: glutathione in reduced form

SD: Sprague-Dawley rats

CEC: circulating endothelial cells

Abstract

Apolipoprotein A1 mimetic peptide (D-4F), synthesized from D-amino acid, enhances the ability of high-density lipoprotein (HDL) to protect low-density lipoprotein (LDL) against oxidation in atherosclerotic disease. Using a rat model of Type I diabetes, we investigated whether chronic use of D-4F would lead to upregulation of HO-1, $CD31^+$ and thrombomodulin (TM) expression, and increase the number of endothelial progenitor cells (EPC). Sprague-Dawley rats were rendered diabetic with streptozotocin (STZ) and either D-4F or vehicle was administered, by intraperitoneal injection, daily for 6 weeks (100 µg/100 g body wt). HO activity was measured in liver, kidney, heart and aorta. After 6 weeks of D-4F treatment, HO activity significantly increased in the heart and aorta by 29 and 31% ($p<0.05$ and $p<0.49$), respectively. Long-term D-4F treatment also caused a significant increase in TM and $CD31^+$ expression. D-4F administration increased antioxidant capacity, as reflected by the decrease in oxidized protein and oxidized LDL, and enhanced EPC function and/or repair, as evidenced by the increase in EPC eNOS and prevention of vascular TM and $CD31^+$ loss. In conclusion, HO-1 and eNOS are relevant targets for D-4F and may contribute to the D-4F-mediated increase in TM and $CD31^+$, the antioxidant and anti-inflammatory properties, and confers robust vascular protection in this animal model of Type 1 diabetes.

Introduction

The development of the 4F peptides has previously been recorded in detail (Navab, et al., 2005b). The major protein in HDL is apolipoprotein A-I (apoA-I). It contains 243 amino acids. Based on the ability to form helixes similar to those in human apoA-I, Anantharamaiah and Segrest searched for peptides smaller than apoA-I. They found that an 18-amino acid peptide with the sequence D-W-L-K-A-F-Y-D-K-V-A-E-K-L-K-E-A-F, which does not have any sequence homology with apoA-I, formed a class A amphipathic helix similar to those found in apoA-I. They named this peptide 18A (Anantharamaiah, et al., 1985). The peptide 18A mimicked many of the lipid binding properties of apoA-I (Anantharamaiah, 1986). The lipid binding characteristics of 18A were improved by blocking the terminal charges (Ac-18A-NH_2) and this modified improved peptide was named 2F to denote that it contains two phenylalanine residues, one each at positions 6 and 18 (Venkatachalapathi, et al., 1993;Venkatachalapathi, et al., 1993). Unfortunately, despite the ability to bind lipids similar to human apoA-I, 2F did not reduce lesions in a mouse model of atherosclerosis (Datta, et al., 2001). It was found that the best predictor of anti-inflammatory and anti-atherosclerosis activity was the ability of peptides to inhibit the induction of monocyte chemotactic activity in a culture of human aortic cells (Datta, et al., 2001). Two peptides that were particularly potent in this assay were tested in mouse models of atherosclerosis, 5F and 4F, which contained 5 and 4 phenylalanine residues on the hydrophobic face of the peptides, respectively. The peptide 5F (Ac-D-W-L-K-A-F-Y-D-K-V-F-E-K-F-K-E-F-F-NH_2) when injected into mice given an atherogenic diet significantly inhibited lesion formation (Garber, et al., 2001). The 4F peptide (Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH_2)

when synthesized from all D-amino acids (D-4F) was found to be suitable for oral administration to mouse models of atherosclerosis and significantly inhibited lesion formation in young mice (Navab, et al., 2002). When D-4F was given orally together with pravastatin there was substantial synergy and regression of lesions was also found (Navab, et al., 2005a).

D-4F re-establishes an antioxidant and anti-inflammatory phenotype through restoration of the balance between nitric oxide (NO) and superoxide (O_2^-) production (Ou, et al., 2003;Ou, et al., 2005), which results in an improvement in vascular function (Rodella, et al., 2006;Ou, et al., 2005). Thus, D-4F decreases endothelial cell (EC) sloughing and apoptosis and restores vascular EC function (Rodella, et al., 2006), although a D-4F effect causing an increase in vascular repair has not been excluded.

Endothelial cell dysfunction, demonstrated by the reduced expression of $CD31^+$ and/or thrombomodulin (TM) (Sandusky, et al., 2002), has been reported within atherosclerotic blood vessels. A $CD31^+$ gene abnormality has also been implicated in the pathogenesis of both atherosclerosis and myocardial infarction (MI). Furthermore, a reduction in plasma TM has also been associated with an increased risk of MI (Morange, et al., 2004). Conversely, increased expression of TM has been shown to limit thrombus formation as well as neointimal growth (Waugh, et al., 2000). Diabetes mellitus (DM) is a major risk factor in the development of atherosclerotic heart disease. The hyperglycemia-mediated generation of reactive oxygen species (ROS) and advanced glycosylation end products accelerate the formation of atherosclerotic lesions (Aronson and Rayfield, 2002), contributing to the pathogenesis of multiple vascular complications (Rodella, et al., 2006;Da Ros, et al., 2004;Aronson and Rayfield, 2002).

Type 1 diabetes has also been shown to reduce both the number and function of bone marrow-derived endothelial progenitor cells (EPC) (Loomans, et al., 2004). This could potentially contribute to the formation of atherosclerotic disease. There is growing evidence to suggest that proper vascular function relies not only on mature EC, but also on EPC (Asahara, et al., 1997). EPC have been shown to contribute to vascular remodeling in atherosclerosis (Sata, et al., 2002) and other cardiovascular diseases (Rafii and Lyden, 2003). More recently, HDL has been shown to provide vascular protection by increasing EPC in apolipoprotein E-deficient mice (Werner, et al., 2005).

The recognition that HO-1 is strongly induced by its substrate heme and by oxidant stress, in conjunction with the robust ability of HO-1 to protect against oxidative insult in cardiovascular disease, suggests that HO-1 may be a target for pharmacological drugs in the alleviation of vascular diseases. The antioxidant effects of HO-1 arise from its capacity to degrade the heme moiety from destabilized heme proteins (Nath, et al., 2000) and to generate biliverdin and bilirubin, which are products of HO, which possess potent antioxidant properties. CO, an HO product as well, is not an antioxidant (Wiesel, et al., 2000) but can cause the induction of antioxidant genes, decrease O_2^- levels and increase GSH levels (Abraham and Kappas, 2005). HO-1-derived bilirubin has also been shown to display cytoprotective properties in the cardiovascular system (Clark, et al., 2000). Numerous reports indicate that higher serum bilirubin levels are associated with a decrease in the risk for coronary artery disease in humans (Vitek, et al., 2002). We, and others, have previously shown that D-4F has a beneficial effect on vascular function (Rodella, et al., 2006); however, the exact mechanism is not known.

The present study explores whether chronic D-4F administration leads to an increase in HO-1 activity specifically relevant to vascular cytoprotection, such as in the heart and aorta. We also investigated the effect of D-4F on the expression of both $CD31^+$ and TM, markers for the onset of atherosclerosis, and on EPC numbers and function in an animal model of diabetes. We demonstrate, for the first time, that D-4F, by increasing HO-1 and eNOS and decreasing circulating oxidants, protected EPC function and increased the expression of $CD31^+$ and TM. These data highlight the chronic effect of daily administration of D-4F in preventing vascular damage, rendering endothelial cells resistant to oxidants in this model of Type 1 diabetes.

Methods

Animal treatment: Male Sprague-Dawley (SD) rats (Charles River Lab, Wilmington, MA), weighing 170–190 g, were maintained on standard rat diet and tap water ad libitum. After rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (65 mg/kg body weight), diabetes was induced by a single injection, via the tail vein, of streptozotocin (STZ, Sigma, St. Louis, MO) (45 mg/kg body weight) dissolved in 0.05 mol/L citrate buffer (pH 4.5). Blood glucose levels were elevated (410+35 mg/dl) two days after the injection of STZ, but were maintained between 240-320 mg/dl in all STZ-treated rats for the 6-week duration of the study by the administration of insulin (NPH 40-60 U/day/kg). Insulin was essential to assure that ketosis and weight loss were not significant. Glucose monitoring was performed using an automated analyzer (Lifescan Inc., Miligitas, CA). D-4F was given as a daily i.p. injection (100 µg/100 g body weight) for 6 weeks, beginning the day after the injection of STZ or sodium citrate buffer (in control rats). Four groups of rats were used: control, STZ alone, STZ plus D-4F, and D-4F alone. The Animal Care and Use Committee of New York Medical College approved all experiments.

Tissue preparation for ultrastructural analysis: Aorta segments were removed and immediately fixed in 2% glutaraldehyde in phosphate buffer (pH 7.4). After 12 hours, the specimens were washed in phosphate buffer, stained with uranyl acetate, dehydrated in decreasing acetone concentrations and embedded in Araldite. Semi-thin (1.5 µm thick) sections were cut by an ultramicrotome and stained with toluidine blue for light microscope observation and to identify the area for the ultrastructural analysis.

Sections were then cut and observed by a Philips CM10 transmission electron microscope.

Detection and quantification of EPC in peripheral blood: Peripheral blood specimens were layered 1:1 onto a Ficoll-Paque Plus (GE Healthcare, Waukesha, WI) and centrifuged at room temperature for 35 minutes at 450 g. The mononuclear cell layer was removed and washed three times with PBS. Following the third wash, cells were suspended in 500 µl PBS, containing anti-RECA-1 (Novus Biologicals, Littleton, and CO) and and anti-$CD34^+$ FITC conjugated antibody $CD34^+$ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). FITC conjugated normal mouse IgG (Santa Cruz Biotechnology) was used as a negative control as described previously (Abraham, et al., 2003;Rodella, et al., 2006).

Effect of hyperglycemia on EPC: The ability of bone marrow-derived cells to differentiate was quantified to determine the effects of diabetes and D-4F on EPC formation. Bone marrow hematopoietic colonies were prepared in methylcellulose cultures as described previously (Lutton, et al., 1993), and grown in the presence of VEGF (100 nM) to induce differentiation into EPC. Bone marrow from control rats (10^6 cells) was cultured at 37°C for 5-14 days. Additional cultures utilized bone marrow from rats treated *in vivo* with STZ and/or D-4F using the same technique.

Immunohistochemical analysis: Aorta segments were collected and fixed in 4% buffered formalin, cut by cryostat (5 µm thick) and stained for the EC markers, $CD31^+$ and TM, using the Avidin-biotin-peroxidase method. Briefly, the sections were incubated with 3% hydrogen peroxide to quench endogenous peroxidase activity. The sections were then incubated for 1 hour at room temperature with monoclonal

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antibodies to detect $CD31^+$ (Pharmingen, Franklin Lakes, NJ) or TM (Labvision Corp., Fremont, CA). Primary antibody incubation was followed sequentially by biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) for 30 minutes then by ABC complex (Vector Laboratories, CA). Negative controls were obtained by omitting the immune serum as a substitute for the primary antibody. Diaminobenzidine was used as chromogen and hematoxylin was used as a nuclear counterstain.

Protein analysis and HO activity: Heart, aorta, liver, kidney and EPC homogenates were used to measure HO activity as described previously (Rodella, et al., 2006). Western blot analysis of tissues or EPC cell homogenates was carried out to determine HO-1, HO-2 and eNOS protein expression (Abraham, et al., 2003;Rodella, et al., 2006). Protein levels were visualized by immunoblotting with antibodies against rat HO-1/HO-2 (Stressgen Biotechnologies Corp., Victoria, BC) and eNOS (Santa Cruz Biotechnology). Briefly, 20 µg of lysate supernatant was separated by 12% SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane, and chemiluminescence detection was performed with the Amersham ECL detection kit according to the manufacturer's instructions (Amersham, Inc., Piscataway, NJ).

Measurement of oxidative stress: Serum samples were collected from untreated and D-4F-treated diabetic and control rats for assessment of oxidative stress. Oxidized proteins (Cayman Chemical Co., Ann Arbor, MI) and LDL (Ox-LDL, Northwest Life Science Specialties, Vancouver, WA) were assayed using ELISA kits according to the manufacturer's instructions.

Statistical analyses: Data are presented as mean ± standard error (SE) for the number of experiments. Statistical significance ($p<0.05$) between experimental groups

was determined by the Fisher method of analysis of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by a single-factor ANOVA for multiple groups or unpaired *t* test for two groups.

Results

Effect of D-4F on glucose levels: As seen in Figure 1, D-4F had no effect on glucose levels. Glucose levels were maintained between 240-320 mg/dl to prevent weight loss. Insulin dose was individualized and thus dosages were different within the same group and were not significantly different between the groups. Under these conditions, body weight did not significantly change (Figure 1B).

Effect of D-4F on HO activity: As seen in Figure 2A-D, HO activity was significantly increased in the heart and aorta in D-4F treated diabetic rats compared to organs obtained from untreated diabetic rats. Six weeks of D-4F treatment resulted in a significant increase in HO activity in the heart, n=3, ($p<0.05$). A similar increase was seen in aortic HO activity ($p<0.01$). No significant increases in HO activity were observed in the kidney or liver after treatment with D-4F (Figure 2B and C, respectively).

Effect of D-4F on EC sloughing: The protective effect of D-4F on the vasculature was demonstrated by the direct quantification of circulating endothelial cells (CEC) in peripheral blood and confirmed by FACS analysis. The number of CEC in peripheral blood was significantly elevated in diabetic rats compared to control animals (50±6, and 4±3 cells/ml peripheral blood). Treatment with D-4F did not affect EC sloughing in control rats. However, in diabetic rats, D-4F attenuated EC sloughing to 20±3 (cells/ml peripheral blood, $p<0.003$ vs. untreated diabetics, n= 13). FACS analysis was used to confirm the increase in CEC in diabetic rats and the reduction in CEC after D-4F treatment (Figure 3). Long-term treatment with D-4F caused a significant decrease in CEC (*$p<0.05$ vs. untreated diabetic rats), promoting endothelial cell survival.

Effect of D-4F on CD31$^+$ and TM expression: Since D-4F had a beneficial effect on heart and aortic HO-1, we examined whether D-4F affected CD31$^+$ and TM. Immunohistochemical staining for CD31$^+$ (Figure 4) and TM (Figure 5) was conducted in aorta isolated from untreated and D-4F-treated diabetic rats. Staining appeared brown and was localized within the EC cytoplasm. In control animals, strong CD31$^+$ immunoreactivity was seen in the aorta (Figure 4A). In diabetic animals, CD31$^+$ staining was either weak or absent (Figure 4B); however, treatment with D-4F restored the pattern to that seen in controls (Figure 4C and D). TM staining was strong in the intima of control rats (Figure 5A) while diabetic rats demonstrated moderate to weak staining (Figure 5B). D-4F treatment restored TM expression in diabetic rats to the level of staining seen in controls (Figure 5C and D). Optical density analysis of immunohistochemical staining provided quantification of the changes in both CD31$^+$ (Figure 4D) and TM (Figure 5D) expression.

Effect of D-4F on EPC function: The effect of D-4F on EPC function was assayed in diabetic rats untreated or chronically administered D-4F (Figure 6). STZ-induced diabetes reduced the formation of EPC colonies from 19.3±1.3 colonies in controls to 8.8±1.3 ($p<0.001$). In diabetic rats treated with D-4F, the number of EPC colonies improved to 17.3±1.5 ($p<0.002$ vs. STZ alone), approaching the level found in control animals.

Effect of D-4F on HO-1 and eNOS in EPC: Since D-4F increased EPC function, as seen by the restoration in TM and CD31$^+$, we examined the effect of D-4F on the levels of HO-1, HO-2 and eNOS in EPC after 10 days of culture. Figure 7A shows the changes in HO-1 (HO-2 is constitutively expressed and was unchanged) and eNOS

levels in response to STZ-induced diabetes and administration of D-4F. The effect of STZ-diabetes was to downregulate HO-1 protein expression. Optical density analysis of eNOS, expressed as a ratio to α-actin, revealed diminished levels of eNOS (0.17±0.04) in STZ-treated rats compared to controls. D-4F treatment produced eNOS expression in diabetic rats (0.30±0.03, $p<0.05$) similar to that in controls (Figure 7B). Treatment with D-4F prevented the loss of HO-1 protein expression to 0.19±0.02 ($p<0.02$), a level consistent with controls (Figure 7C).

HO activity was 167.7±21.3 pmol bilirubin formed/mg protein in EPC obtained from diabetic rats compared to 268.7±35.5 pmol bilirubin formed/mg protein in controls ($p<0.05$). D-4F treatment increased HO activity to 317.3±23.7 pmol bilirubin formed/mg protein ($p<0.005$) in diabetic rats, but did not significantly affect HO activity in control rats (Figure 7D).

Effect of D-4F on serum oxidative stress: The effects of STZ and D-4F on the levels of oxidative stress were assayed using ELISA for oxidized proteins (Figure 8A) and LDL (Figure 8B). Oxidized protein (carbonyl) content was elevated in diabetic rats (1.62±0.36 nmol/mg) compared to controls (1.20±0.13 nmol/mg, $p<0.01$). D-4F attenuated this increase ($p<0.05$ vs. untreated diabetic) in carbonyl content (1.33±0.19 nmol/mg). The level of pro-atherogenic oxidized-LDL was elevated in diabetic rats (11.76±0.82 units/l) compared to controls (8.12±1.47 units/l, $p<0.02$). D-4F reduced the level of oxidized-LDL ($p<0.05$ vs. untreated diabetic rats) to 9.18±1.06 (units/l), a level consistent with that found in controls. These results suggest that D-4F has a beneficial effect on the vascular system, preventing oxidative stress and restoring EPC function.

Discussion

We have demonstrated, in this study, that daily administration of D-4F prevents the loss of EPC function and contributes to vascular repair in diabetic rats. Four observations support this conclusion. First, D-4F selectively increased HO-1 expression in the aorta and heart, an observation not seen in the liver or kidney. Second, the increases in HO-1-derived CO and bilirubin in EPC, following the upregulation of HO-1 via D-4F administration, paralleled the increases in TM and $CD31^+$ expression. HO-1 derived CO and bilirubin have been shown to prevent endothelial cell death and apoptosis both *in vitro* and *in vivo* (Abraham, et al., 2003;Rodella, et al., 2006;Pileggi, et al., 2001;Ye and Laychock, 1998). Third, increases in ox-LDL and oxidized proteins were prevented by chronic administration of D-4F. The hyperglycemia-mediated increases in ROS generation and O_2^- production contribute to vascular endothelial cell dysfunction and apoptosis (Rodella, et al., 2006;Turkseven, et al., 2005), and have been shown to be reversed by the induction of HO-1 (Rodella, et al., 2006;Turkseven, et al., 2005). Fourth, chronic administration of D-4F changed the diabetic EPC from a naïve to a defensive phenotype by producing a robust increase in eNOS and HO-1, as reflected by an increase in EPC function in culture and *in vivo.* The salutary effect of D-4F was reflected by the increases in TM and $CD31^+$ expression. Reduced EC expression of $CD31^+$ and TM, an important indicator of endothelial cell death, is associated with the progression of atherosclerotic heart disease (Sandusky, et al., 2002), while restoration of their expression prevents atherosclerosis and myocardial infarction (Morange, et al., 2004).

Our results are in agreement with previous studies showing that the downregulation of TM expression in EC occurs in coronary atherosclerosis in humans. Prevention of the diabetes-induced decrease in EC expression of $CD31^+$ and TM provides an insight into the mechanism(s) of the anti-atherosclerotic properties of D-4F (Navab, et al., 2004a;Navab, et al., 2004b). The D-4F-mediated increase in $CD31^+$ and TM expression in the aorta of diabetic rats suggests that an increase in EPC function, leading to the repair of the endothelium, may be a contributing factor to the increases in TM and $CD31^+$.

Our results do not distinguish whether D-4F caused an increase in existing EC regeneration within the diabetic aorta or whether the increase was due to new EPC function. Regardless of the mechanism, chronic treatment with D-4F caused restoration of both TM and $CD31^+$ and increased vascular repair, which would be considered clinically relevant in diabetes. The increase in TM and $CD31^+$ limits neointima formation and EC dysfunction. The diminished function of vascular EC that occurs with diabetes (Waugh, et al., 2000) is accompanied by a reduction in EPC function (Loomans, et al., 2004), which further impacts the integrity of the intact endothelial lining.

The increases in HO-1 and eNOS, induced by the chronic administration of D-4F, are likely major factors in EPC protection. This is of particular interest because reversal drugs, such as the statins, known for their anti-atherosclerotic properties, have been shown to increase both HO-1 and eNOS. Statins have a strong positive effect on HO-1 protein (Grosser, et al., 2004) and eNOS (Li and Mehta, 2003) expression, and reduce in adhesion molecules (Li and Mehta, 2003). The increase of HO-1 and eNOS explains the mechanism by which the statins exert antioxidant properties, as seen by the

decrease in oxidized LDL. Therefore, the chronic effects of D-4F administration, with the resulting decrease in oxidized LDL and oxidized proteins, may be attributed to the D-4F-mediated increase in both eNOS and HO-1.

HO-1 is induced under a wide variety of conditions associated with oxidative stress and is regarded as a protective response to oxidants. In the present study, we report that HO-1 and eNOS protein levels were restored in isolated mononuclear cells by chronic D-4F treatment. An increase in HO-1 will increase heme degradation and has the associated beneficial effect of increasing CO and bilirubin, which are important regulators of vascular function. Bilirubin is an important antioxidant in humans and an increase in serum levels prevent cardiovascular disease, as has been seen in Gilbert's disease (Vitek, et al., 2002). HO-1 upregulation also increases the expression of eNOS and superoxide dismutase (Turkseven, et al., 2005), which contribute to the reduction in oxidized protein levels in serum, leading to vascular repair. These results are also in agreement with the reported beneficial effect of eNOS on EPC function (Aicher, et al., 2003).

HO-1 has been reported to be localized within foam cells that contribute to the formation of atherosclerotic lesions (Nakayama, et al., 2001). A decrease in HO activity has been shown to result in the accelerated formation of atherosclerotic lesions in native vessels (Yet, et al., 2003;Ishikawa, et al., 2001a) and vein grafts (Yet, et al., 2003). Induction of HO-1 inhibits the formation of oxidized LDL with the resultant prevention of the formation of atherosclerotic lesions (Ishikawa, et al., 2001b). The fact that D-4F increases the levels of CO and bilirubin as well as eNOS in EPC suggests

that D-4F has a clinically relevant role in reducing pro-atherogenic ox-LDL in diabetic rats and may have an anti-inflammatory effect on the vascular system.

In conclusion, chronic D-4F treatment resulted in modulating the EPC phenotype, as reflected by the increases in HO-1 and eNOS, which may contribute to the increased levels of aortic $CD31^+$ and TM. Therefore, HO-1 and eNOS are considered relevant targets for D-4F. They promote EC cell survival, affording vascular cytoprotection in diabetic animals.

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All authors had full access to the data and take responsibility for its integrity. All authors have read and agree with the manuscript as written.

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Footnotes

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Legends for Figures

Figure 1. **(A)** Serum glucose in control and STZ-treated rats. Diabetes was induced by a single injection of STZ (45 mg/kg bw). **(B)** Total body weight gain or loss for control diabetic rats (n=6).

Figure 2. Effect of D-4F on HO activity in heart **(A)**, kidney **(B)**, liver **(C)** and aorta **(D)**. Rats were chronically treated with or without D-4F as described in Methods (n=3). Results are expressed as the mean ± SE of bilirubin formed mg/60 min. *p<0.05 is significant compared to the corresponding control.

Figure 3. FACS analysis of RECA-1 positive cells (arrows) in the peripheral blood of control (left panel), STZ-induced diabetic (center panel), and D-4F treated diabetic (right panel) rats; *p<0.05 vs. control, †p<0.003 vs. STZ treated (n=13).

Figure 4. Immunohistochemical staining of $CD31^+$ EC (arrows) from control **(A)**, diabetic **(B)** and D-4F treated diabetic rats **(C)**. Optical density analysis **(D)** demonstrates the loss of $CD31^+$ staining in diabetes and preventing its loss by D-4F; *p<0.05 vs. control, †p<0.05 vs. STZ treated; I=Intima; M=Media (n=4).

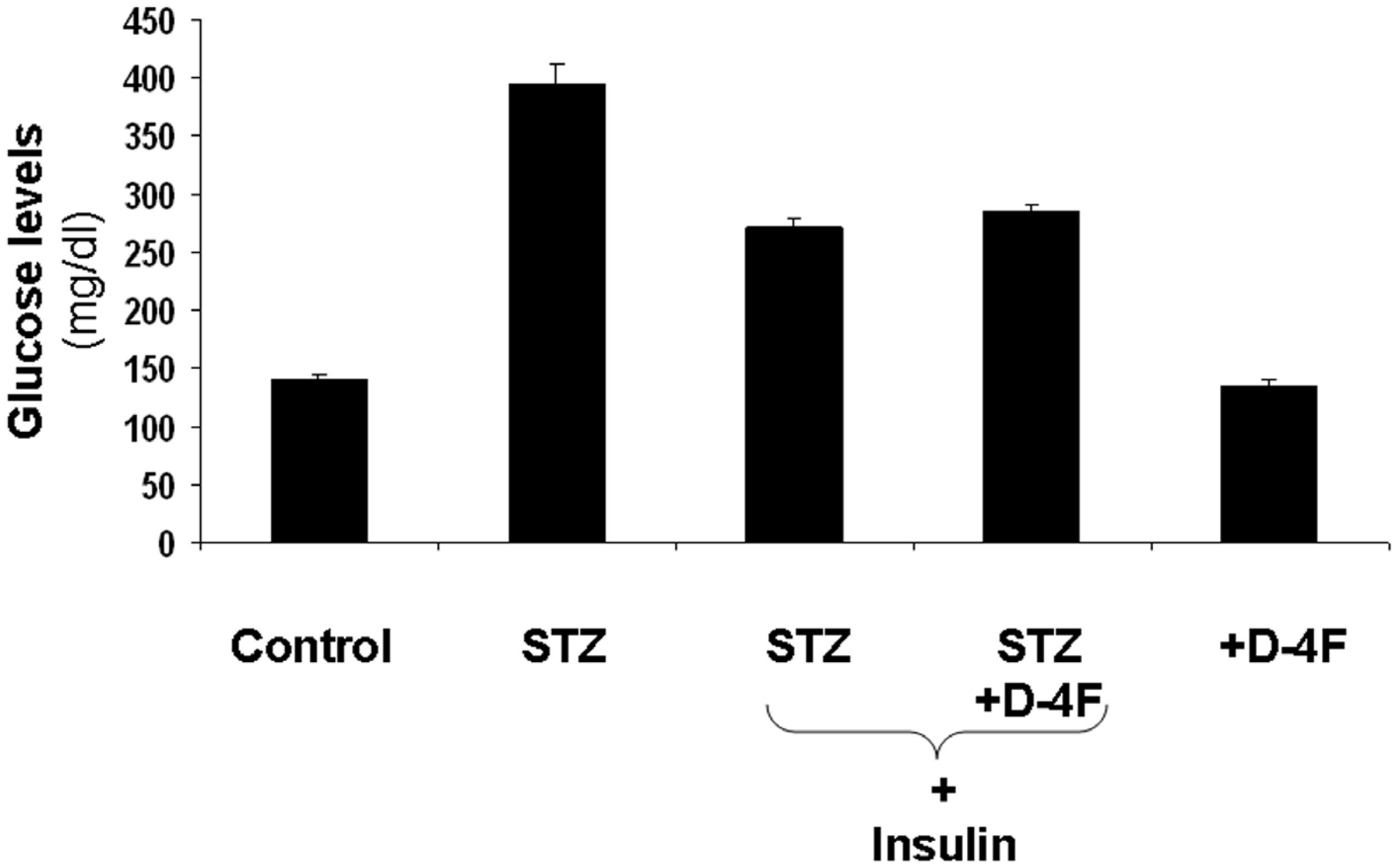
Figure 5. Immunohistochemical staining of TM in EC (arrows) from control **(A)**, diabetic **(B)** and D-4F treated diabetic rats **(C)**. Optical density analysis **(D)** demonstrates the loss of TM staining in diabetes and its restoration by D-4F; *p<0.05 vs. control, †p<0.05 vs. STZ treated; I=Intima; M=Media (n=4).

Figure 6. EPC formation was assayed in *ex vivo* mononuclear cells marrow cultures from control and STZ-treated rats and rats treated with D-4F (n=4); *p<0.001, †p<0.002.

Figure 7. (A) Representative Western blots for eNOS, HO-1, HO-2 and actin in STZ- and D-4F treated rats. **(B)** Optical density of eNOS/ actin (n=3); *p<0.05 vs. control, †p<0.05 vs. STZ. **(C)** HO activity in mononuclear cells taken from control and diabetic rats; (n=3), *p<0.05 vs. control, †p<0.005 vs. STZ treated.

Figure 8. (A) ELISA analysis for the presence of oxidized proteins in the serum of STZ- and D-4F treated rats; (n=3), *p<0.01 vs. control, †p<0.05 vs. STZ treated. **(B)** Presence of pro-atherogenic oxidized LDL was assayed using ELISA; p<0.02 vs. control, †p<0.04 vs. STZ treated.

Figure 1A



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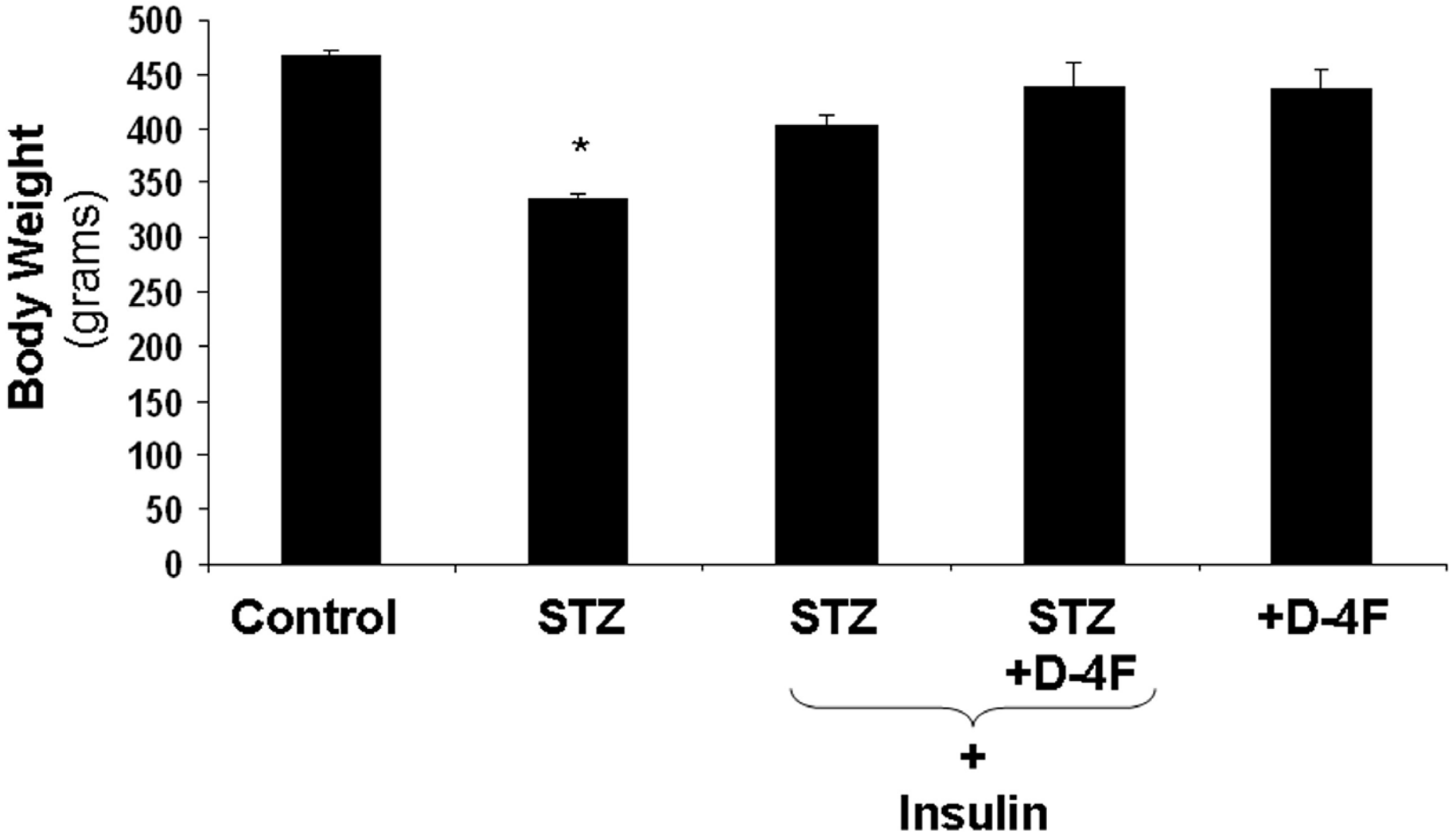
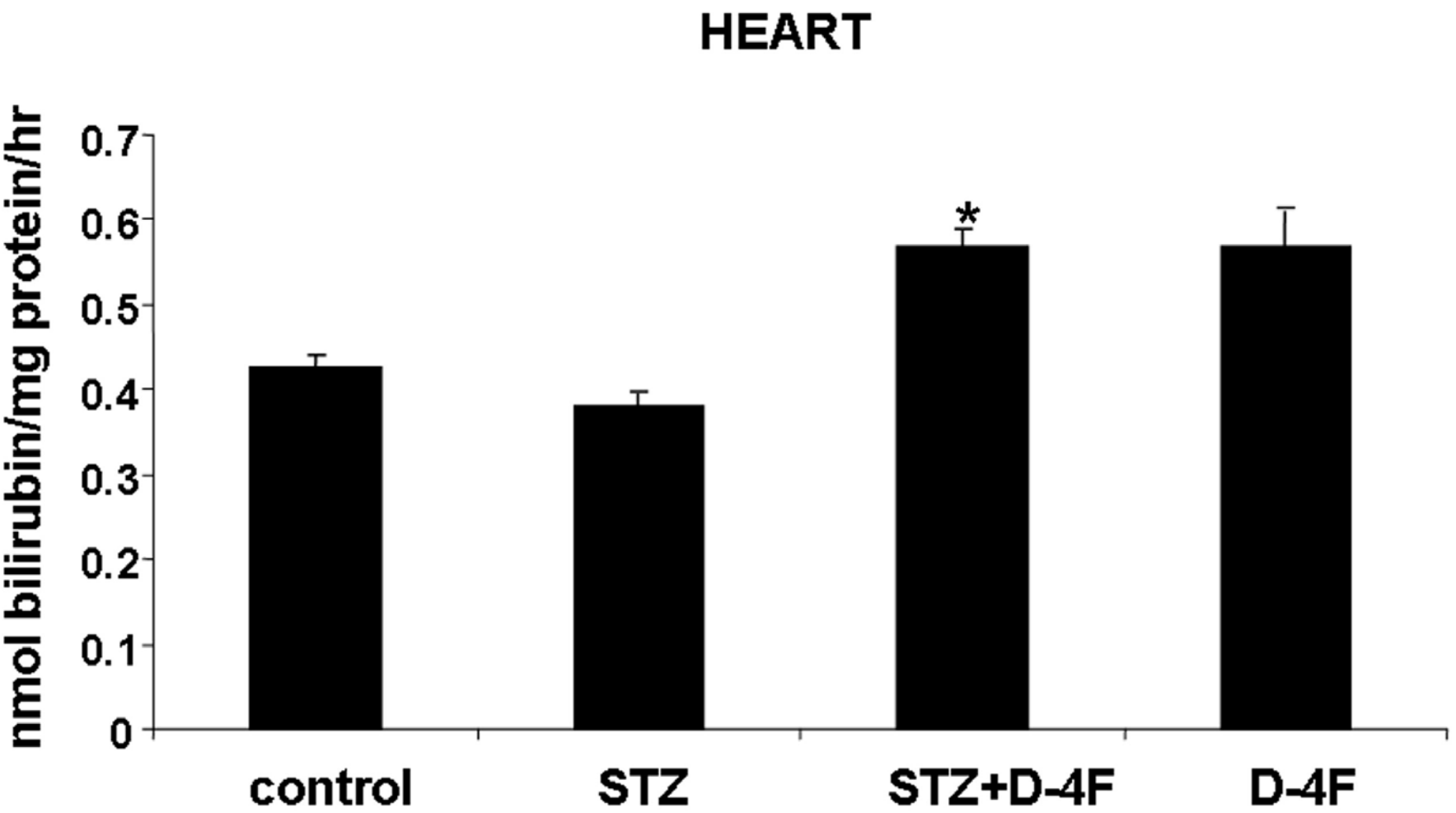


Figure 2 A



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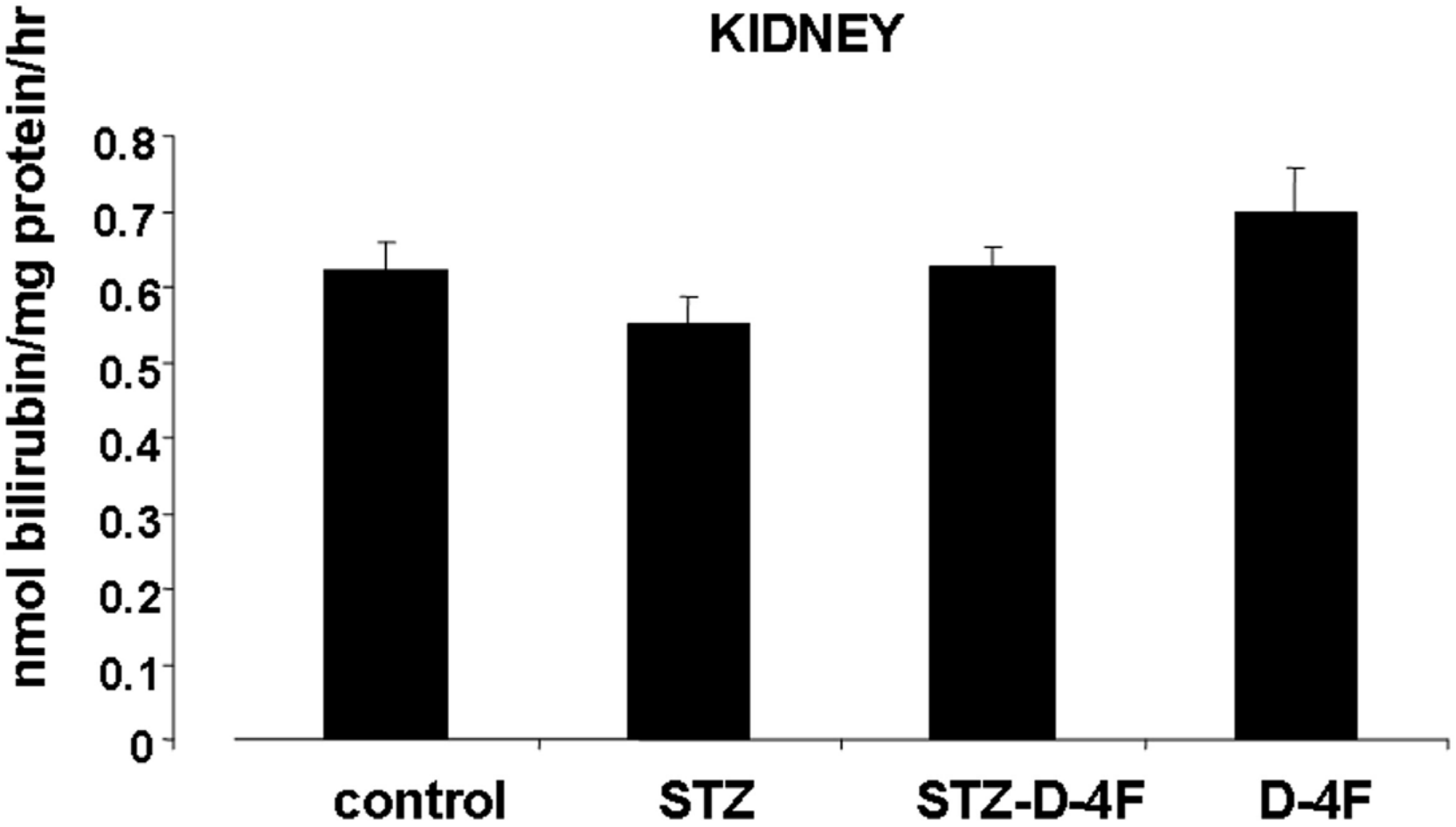
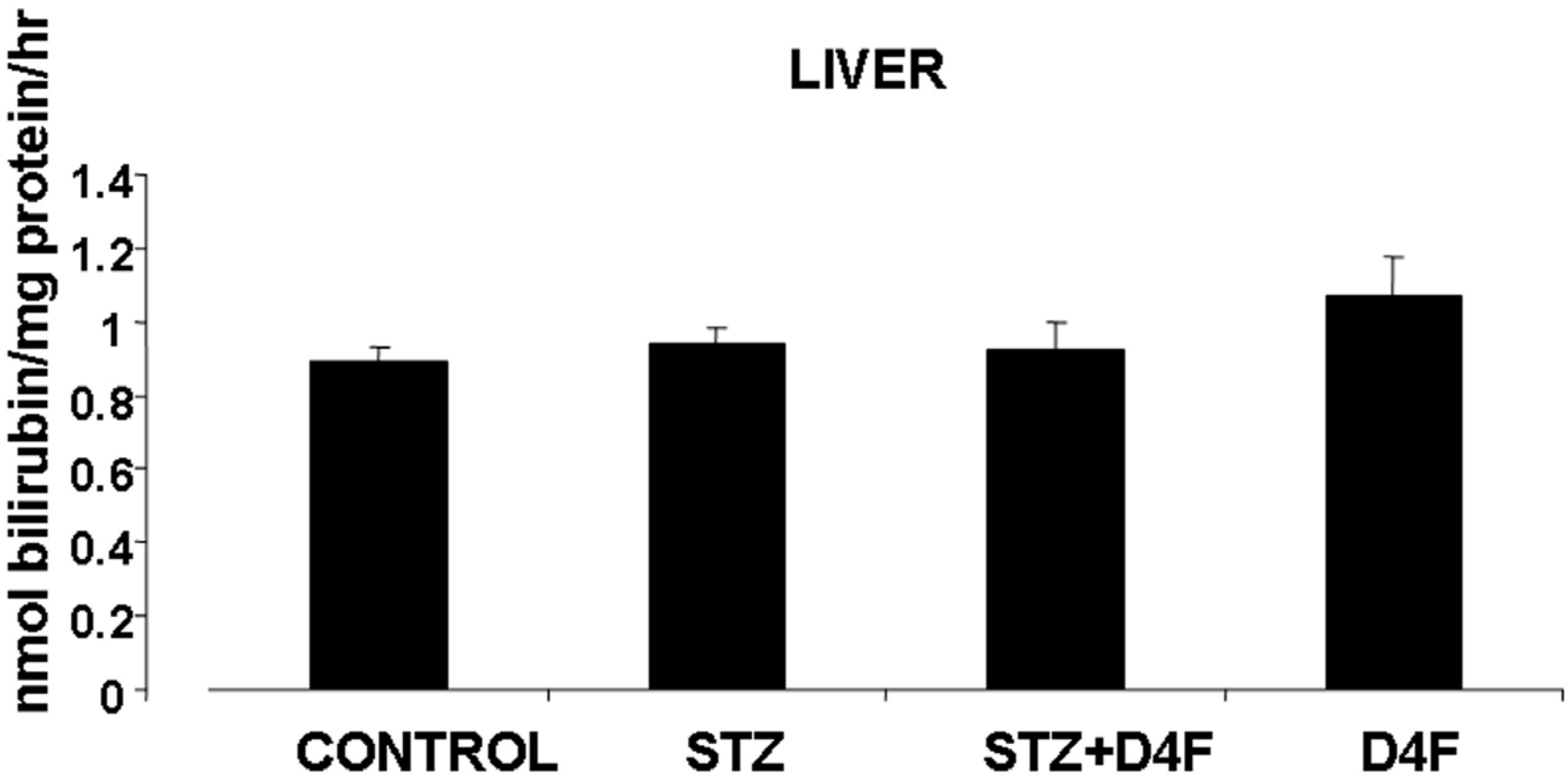


Figure 2C



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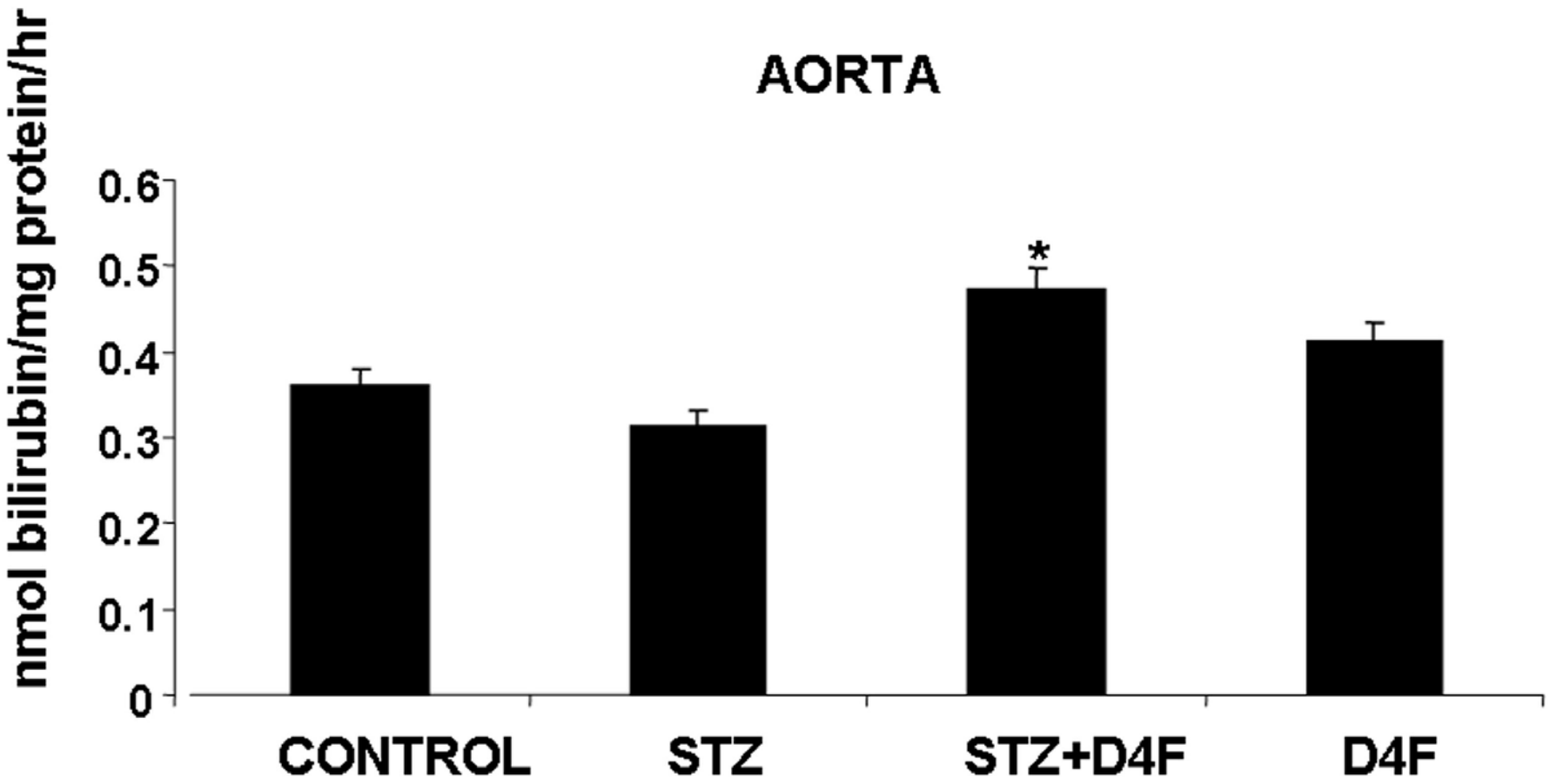

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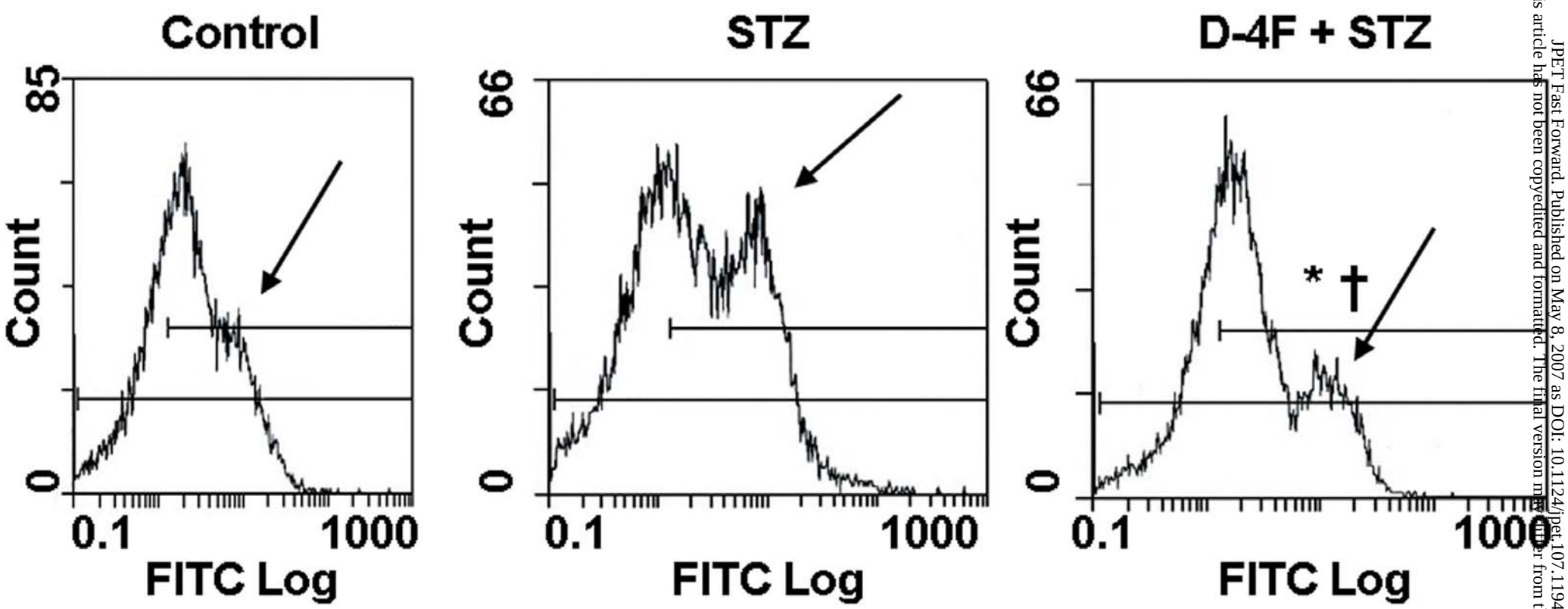

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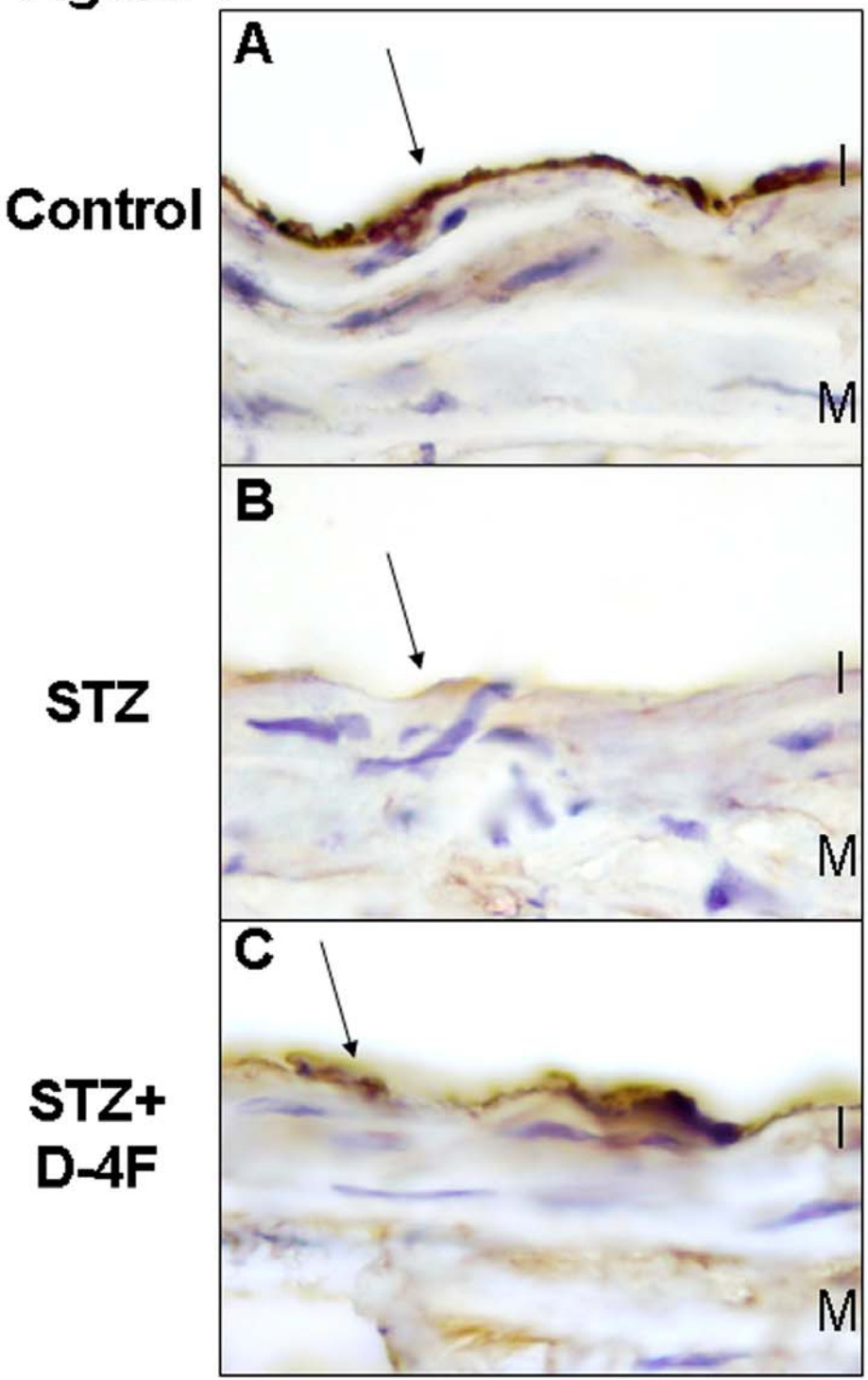


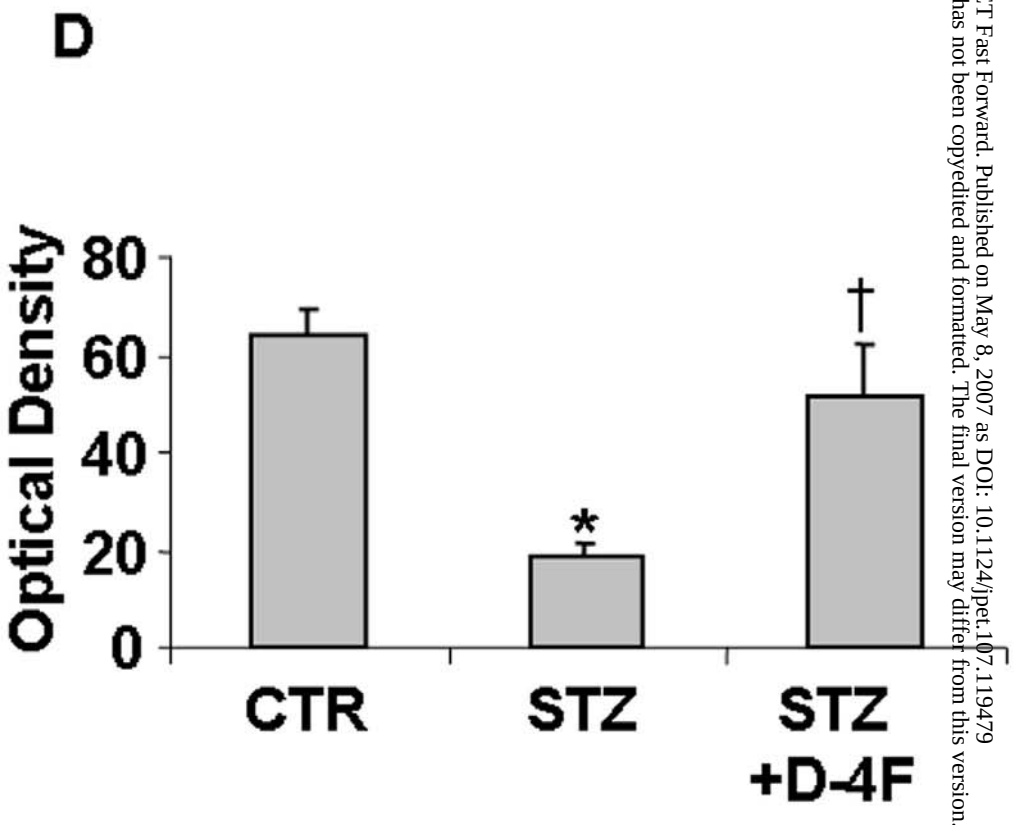


Figure 5

Figure 6

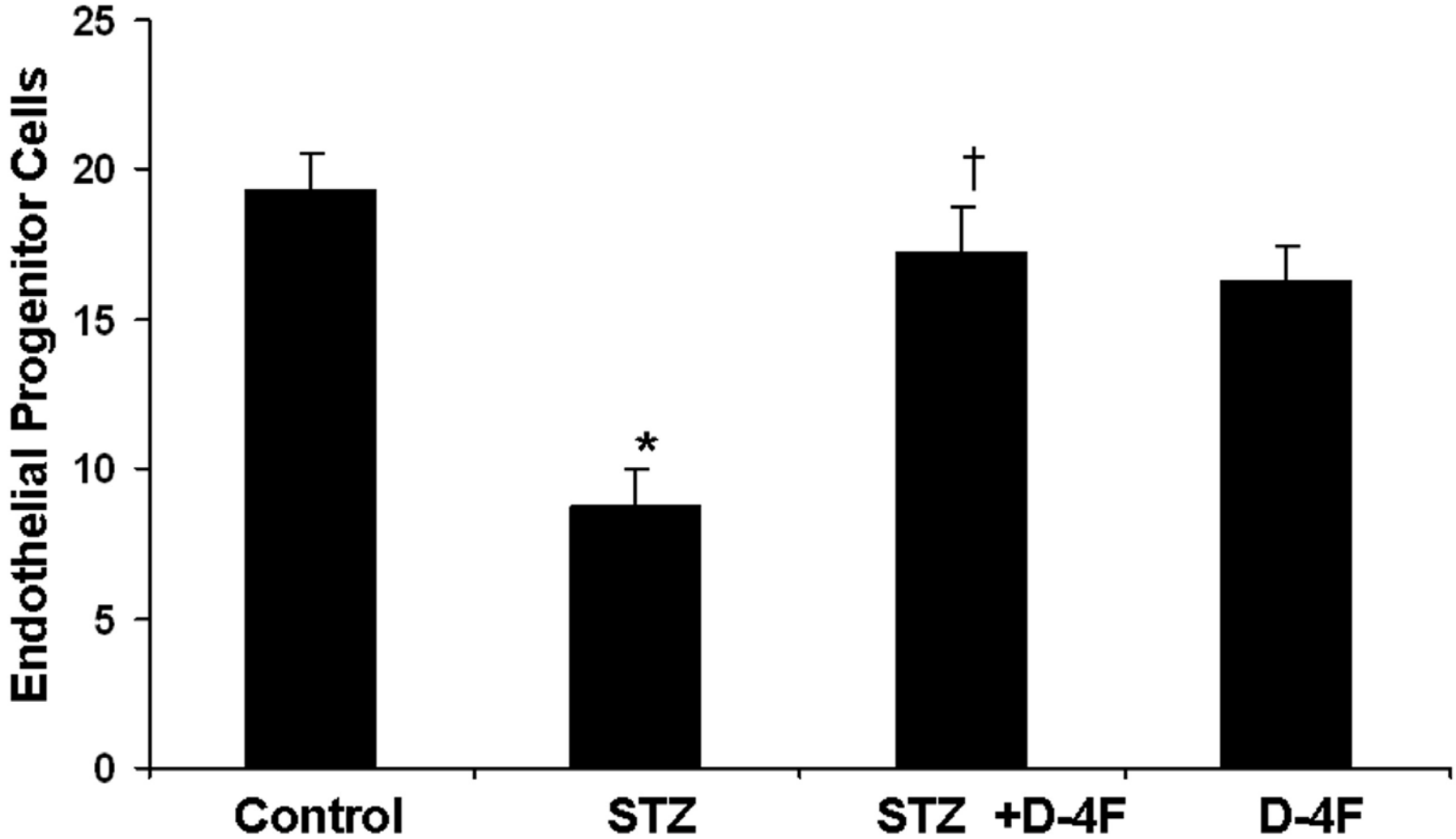
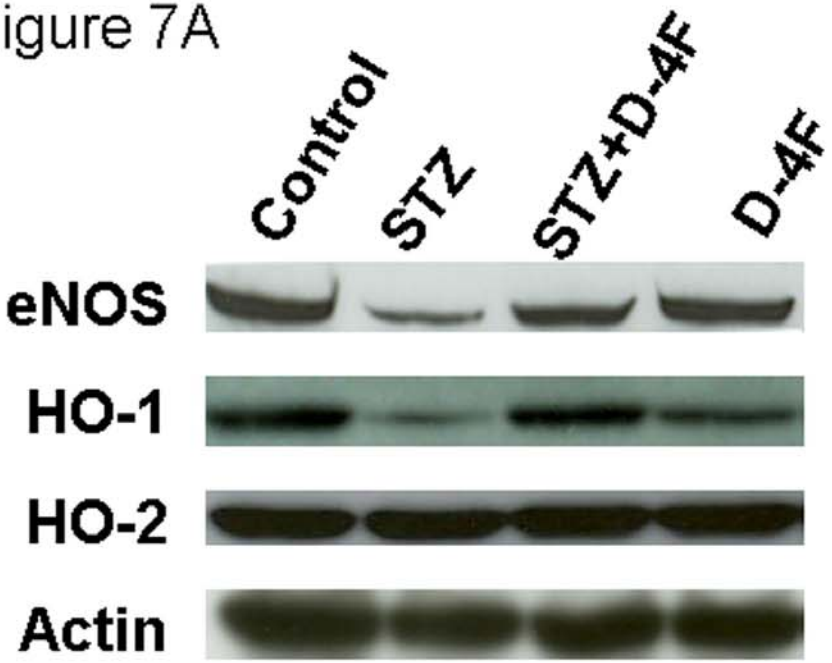


Figure 7A



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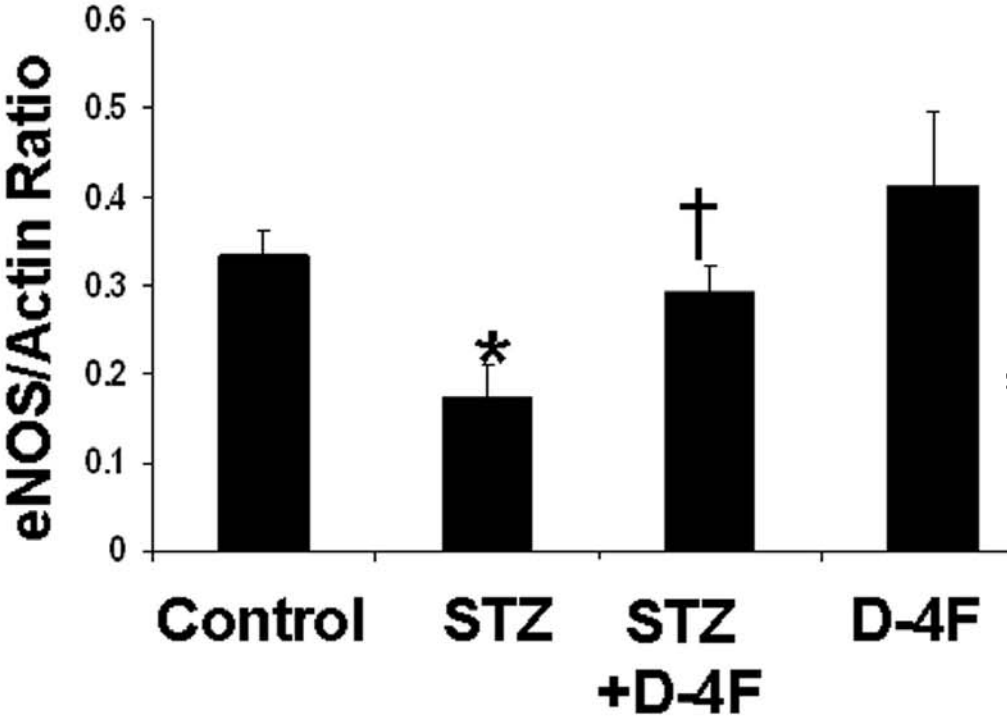

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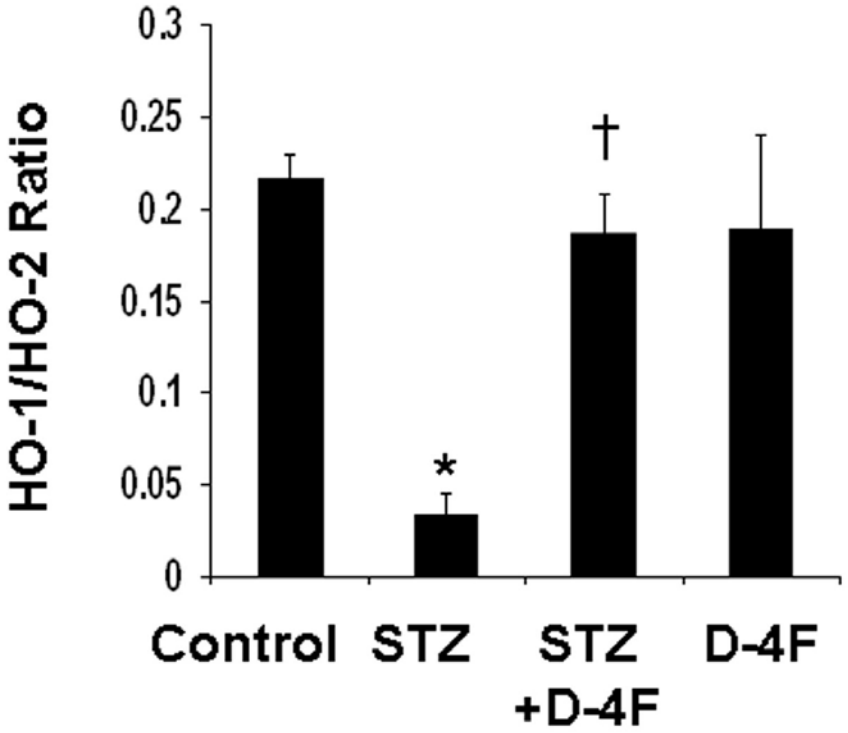

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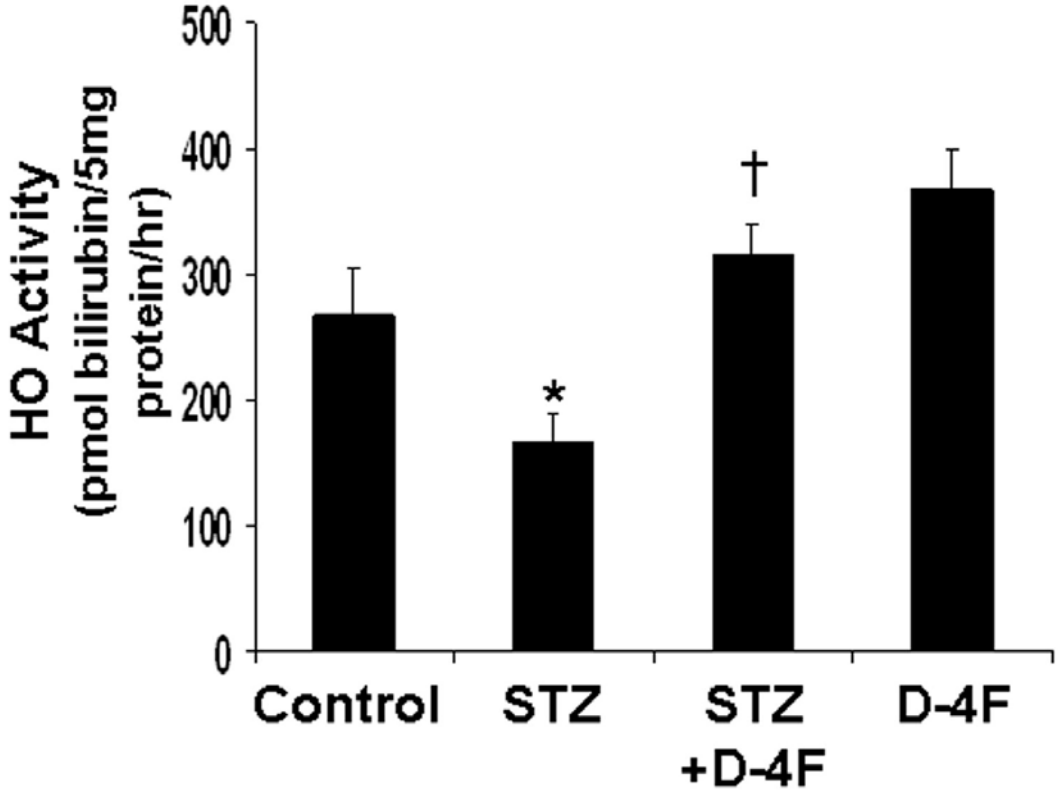

Figure 8