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Changes in the fibrinolytic components of cultured human umbilical vein endothelial cells induced by endotoxin, tumor necrosis factor-α and interleukin-1α

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ABSTRACT

Background and Objective. **Vascular fibrinolysis, a major natural defense mechanism against thrombosis, is a highly regulated process. The aim of this study was to evaluate the effect of endotoxin, tumor necrosis factor-α (TNFα) and interleukin-1α (IL-1α), on the fibrinolytic potential of cultured human umbilical vein endothelial cells (HUVEC).**

Design and Methods. **Samples of stimulated conditioned media were collected over a period of 24 hours to determine: plasminogen activator (PA) and plasminogen activator inhibitor (PAI) activity, PAI-1 mRNA, tissue-type plasminogen activator (t-PA) antigen and urokinase-type plasminogen activator (u-PA) antigen.**

Results. **Similar changes were observed after endotoxin and cytokine stimulation: there was a significant increase of PAI activity ($p<0.01$), starting at 6 hours, which remained 24 hours after stimulation. PAI-1 mRNA also showed an important rise with these agents, although cytokines induced an earlier and more intense inhibitor response (up to 6-fold increase). PA activity increased significantly at 6 hours ($p<0.01$) to drop at 24 hours and was mainly related to the presence of u-PA.**

Interpretation and Conclusions. **We conclude that endotoxin, TNFα and IL-1α induce profound alterations in the fibrinolytic potential of HUVEC, characterized by an initial rise of activators (u-PA) followed by a strong increase of PAI-1. These changes may be of pathophysiologic significance for thrombosis and inflammatory reactions.**

Key words: endothelium, endotoxin, cytokines, PAI, plasminogen activators

Normal hemostasis is the result of a delicate balance between clot formation (blood coagulation) and dissolution (fibrinolysis). Modulation of activity of the fibrinolytic system has been implicated in the pathogenesis of thromboembolic phenomena associated with inflammation.[1]

The fibrinolytic system contains a proenzyme, plasminogen, which is converted to the active enzyme plasmin by the plasminogen activators, tissue-plasminogen activator (t-PA) and urokinase-plasminogen activator (u-PA). Inhibition of the fibrinolytic system may occur either at the level of the plasminogen activators, by specific plasminogen activator inhibitors (mainly PAI-1), or at the level of plasmin, mainly by α_2-antiplasmin.[2]

Vascular endothelial cells play a key role in the regulation of fibrinolytic mechanisms. Cultured human endothelial cells synthesize and secrete fibrinolytic system proteins including t-PA, u-PA and their primary physiologic inhibitor PAI-1.[3-5] Endothelial fibrinolytic potential can be modified by the action of several agents. Endotoxin, a cell wall constituent of Gram negative bacteria, is able to induce a marked increase of tissue factor[6] and PAI-1,[7,8] thus contributing to microthrombosis and multiorgan failure associated with sepsis. On the other hand, exposure of endothelial cells to cytokines such as tumor necrosis factor (TNF) or interleukin-1 (IL-1) shifts the pro/antifibrinolytic balance of these cells resulting in increased synthesis and expression of PAI-1, while suppressing cell-surface anticoagulant activity.[9-11]

The present study was carried out to study the effect of bacterial endotoxin, TNFα and IL-1α on the fibrinolytic potential of human umbilical vein endothelial cells (HUVEC) by measuring the production of t-PA, u-PA and PAI.

Design and Methods

Materials

Medium 199 with Earle's salts (MEM 199) and Hank's balanced salt solution were purchased from BioWhittaker (Belgium); recombinant human TNFα and recombinant human IL-1α, from Amersham (UK); collagenase A from *Clostridium histolyticum* from Boehringer Mannheim (Germany); rabbit polyclonal antibody against human urokinase from American Diagnostica (USA); murine monoclonal antibody against t-PA (MA3B5) was purified in our laboratory as described elsewhere;[12] Dulbecco's phosphate buffered saline (PBS), trypsin-EDTA, serum

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free media endothelial cells (SFMEC), penicillin-streptomycin mixture and L-glutamine from Gibco (UK); bovine gelatine and endotoxin from *Escherichia coli* 0127:B8 from Sigma (USA).

Cell culture

Endothelial cells were isolated from human umbilical cords obtained less than 8 hours after delivery, essentially as described by Jaffe *et al*.[13] with minor modifications. The umbilical vein was cannulated, perfused with PBS and incubated at 37ºC with collagenase A 0.5 mg/mL for 15 min. Cells thus obtained were centrifuged at 250 g for 5 min, resuspended in culture medium (MEM 199 containing 20% pooled human serum, L-glutamine 2 mM, penicillin 50 IU/mL and streptomycin 50 µg/mL) and seeded in 25 cm^2 culture flasks precoated with 0.1% gelatine in PBS (vol/vol). Cultures were incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air. The medium was changed 24 hours after seeding and again after a further 48 hours. Confluent cultures were subcultured at a ratio 1:3 by adding 1 mL trypsin-EDTA at 37ºC for 3 min. We discarded Gram negative bacterial contamination by a *Limulus* assay.[14]

Treatment of HUVEC with endotoxin, TNFα and IL-1α

All studies of HUVEC were performed with confluent cultures on the third passage. Twenty-four hours before stimulation cultures were washed three times with Hank's balanced salt solution after which fresh SFMEC, penicillin 50 IU/mL and streptomycin 50 µg/mL were added. Cultures derived from the same umbilical cord were incubated with endotoxin, TNFα or IL-1α to final concentrations of 1, 10, 100 ng/mL and 1 and 10 µg/mL. Aliquots of culture supernatants were harvested before stimulation (basal samples) and 2, 6 and 24 h. Additional non-stimulated cultures were used as negative controls.

Functional and antigenic assays

PAI activity was measured by a chromogenic assay essentially as described in ref. #15 (Coatest PAI, Chromogenix, Sweden). PA activity was spectrophotometrically determined[16] using a commercially available amidolytic assay (Coatest t-PA, Chromogenix, Sweden). Assays were performed to ascertain whether PA activity was dependent on either t-PA or u-PA by incubating the conditioned media with a molar excess of antibodies which partially block their activity and using pure t-PA and u-PA as controls. t-PA and u-PA antigen were determined by commercially available ELISAs[17,18] (TintElize t-PA and TintElize u-PA from Biopool, Sweden).

Analysis of PAI-1 mRNA

Isolation of mRNAs. Cell mRNA was obtained from cultures stimulated with 100 ng/mL of each agent by hybridizing the polyadenylated tails of mRNA molecules to oligo dT primers coupled to a solid phase matrix (Oligotex TM, Qiagen, Germany).[19] Briefly, confluent cultures were trypsinized and collected as a cell pellet. Lysis was added and cell lysates were homogenized and centrifuged for 3 min at 14,000xg to remove the cell debris and protein. The supernatants were incubated with 2 mg of the oligotex suspension for 10 min at room temperature. The hybrids were washed and the mRNA was eluted by lowering the ionic strength followed by precipitation with 2.5 vol ethanol. The resulting pellet was washed with ethanol 70%, vacuum dried and resuspended in diethyl pirocarbonate-treated water. After determining the concentration spectrophotometrically, the mRNA was stored at –80ºC. Averages of three experiments performed with cells obtained before stimulation and 2, 6 and 24 hours after are reported for each stimulation.

Isolation of PAI-1 cDNA by reverse transcription (RT)-PCR. The reverse transcription reaction was performed in a final volume of 20 µL using 200 U Moloney murine leukemia virus RT (GIBCO BRL), 2 µL RT buffer, 100 ng/µL random hexamers (Boehringer Mannheim), 1 mM dNTPs (Pharmacia), 20 U RNAse inhibitor (Pharmacia), 5 mM DTT (Gibco BRL) and 35 ng of mRNA at 37ºC for 1h.

PCR primer pairs used in this procedure were (5'-ACA GGA GGA GAA ACC CAG CAG-3') and (5'-CCG TCT GAT TTG TGG AAG AGG-3') upstream and downstream respectively giving a PCR product of 434 bp (nucleotides 217-651) from human PAI-1 cDNA.[20] Oligonucleotides (5'-3') d(CCA AGG TCA TCC ATG ACA AC) and d(TGT CAT ACC AGG AAA TGA GC) were used to amplify a 464 bp fragment for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA located between nucleotides 476 and 940.[21] cDNA was amplified in a final volume of 50 µL in the presence of 10 and 20 ng/mL of each primer of PAI-1 and G3PDH respectively and PCR master mix (2 U Taq polymerase from Boehringer Mannheim, 1.5 mM $MgCl_2$, 40 mM KCl, 16 mM Tris-ClH pH 8.3).[22] PCR was performed using the GeneAmp 2400 PCR system (Perkin Elmer) with the following amplification profile: 40 s at 95ºC, then 23 cycles (20 s denaturation at 95ºC; 15 s annealing at 56°C for G3PDH and 58ºC for PAI-1; 15 s extension time at 72ºC) followed by a final extension at 72ºC for 5 min. Fifteen microliters of the reaction mix were electrophoresed in 1.5% agarose gel and amplified bands were visualized by ethidium bromide. PCR band intensities were determined by densitometric analysis with the Gel Doc 1000 UV fluorescent system and Molecular Analyst software for quantification of images (BioRad, USA). Values corresponding to PAI-1 amplification were normalized with those for G3PDH amplification.

PCR conditions were as follows: 15 ng mRNA and 23 cycles for PAI-1 and 20 ng mRNA and 23 cycles for G3PDH. To assess the reproducibility of the assay we calculated the index of intra-assay variation and the

mean coefficient of inter-assay variation in 8 samples (9% and 18% respectively).

Statistical analysis

Results are expressed as mean ± SEM of six experiments in supernatant samples and of three experiments in cell samples. The Mann-Whitney test was used for mean comparisons, and the Spearman rank correlation test to ascertain whether stimulation was dose-dependent. A *p* value <0.05 was considered to be significant.

Results

Effect of endotoxin and cytokines on endothelial PAI-1

Stimulation of HUVEC with increasing amounts of endotoxin, TNFα and IL-1α induced a marked increase of PAI activity (Table 1 and Figure 1). The difference was significant at 24 hours in the case of endotoxin stimulation (reaching 200% in relation to control cultures with higher doses), and at 6 and 24 hours for TNFα and IL-1α stimulation ($p<0.05$). Whereas the lowest dose of endotoxin already induced a significant PAI increase ($p<0.01$ at 24 h), a dose-dependent response was observed after cytokine stimulation.

A progressive increase in the level of PAI-1 gene expression was also observed after endotoxin stimulation, reaching 220% at 24 h with a dose of 100 ng/mL. An earlier and more significant increase was observed at 2 and 6 h after cytokine stimulation (280% with TNF and 600% with IL-1) and then decreased at 24 h (Figure 2).To assess whether the PAI-1 mRNA increase was due to new protein synthesis, additional experiments were performed in stimulated HUVEC incubated with cycloheximide (protein synthesis inhibitor); no changes in mRNA were detected.

Effect of endotoxin and cytokines on endothelial PA

A dose-dependent increase of PA activity was found in the conditioned media of HUVEC stimulated with all agents, starting 2 hours after stimulation with TNFα or IL-1α in a range of 100 ng/mL to 10 µg/mL ($p< 0.01$) and 6 hours for endotoxin in a range of 10 ng/mL to 10 µg/mL ($p<0.01$) decreasing at 24 h (Table 2 and Figure 3). Maximum values were obtained at 6 hours. The strongest effect was observed after addition of IL-1α (1 µg/mL), which resulted in a 14-fold increase with respect to control cultures.

In order to determine which of the plasminogen activators would contribute to the observed increased PA activity, a series of experiments was performed using specific antibodies and measuring the t-PA and u-PA protein concentrations in the supernatants. The incubation of stimulated samples (100 ng/mL of endotoxin or cytokines) with a molar excess of an antibody against either u-PA or t-PA resulted in PA inhibition only in the presence of u-PA. While no differences in t-PA antigen between stimulated and control cultures were observed, a dose-dependent increase of u-PA levels was observed 6 hours after stimulation with all agents in concentrations from 100 ng/mL to 10 µg/mL ($p<0.01$), reaching maximum values after IL-1α stimulation, then decreasing by 24 h (Figure 4). These results indicate that u-PA was the main activator responsible for the increased PA activity.

Table 1. Effect of HUVEC stimulation with different concentrations of endotoxin, TNFα and IL-1α on PAI activity measured in the conditioned media. Mean ± SEM are reported.

Endotoxin						
PAI activity (AU/10^5 cells)	*Control*	*1 ng/mL*	*10 ng/mL*	*100 ng/mL*	*1 µg/mL*	*10 µg/mL*
Basal	21.4±2.0	22.1±3.1	22.8±1.4	25.5±4.2	23.6±2.9	25.5±4.1
2h	28.1±2.2	29.7±2.4	27.9±2.8	28.8±3.7	32.6±4.3	27.8±4.4
6h	28.3±2.9	35.4±2.2	34.5±3.5	33.3±2.2	34.9±3.6	35.5±3.5
24h	25.5±2.8	41.9±1.5°	41.9±1.8°	44.1±1.9°	46.9±0.5°	44.4±1.6°
TNFα						
PAI activity (AU/10^5 cells)	*Control*	*1 ng/mL*	*10 ng/mL*	*100 ng/mL*	*1 µg/mL*	*10 µg/mL*
Basal	26.7±5.3	27.1±5.0	26.7±5.0	28.8±4.2	27.8±3.9	26.7±4.2
2h	29.3±3.9	32.9±3.2	33.1±3.3	33.1±4.5	32.7±3.5	31.2±3.7
6h	25.9±4.4	33.3±5.5	36.7±3.4	42.0±4.2*	41.8±4.5	38.1±3.5
24h	27.1±3.1	36.4±1.7*	34.7±1.9	41.2±3.2*	38.0±3.4*	39.1±4.1*
IL-1α						
PAI activity (AU/10^5 cells)	*Control*	*1 ng/mL*	*10 ng/mL*	*100 ng/mL*	*1 µg/mL*	*10 µg/mL*
Basal	29.6±3.4	32.5±2.8	27.4±3.0	28.4±3.6	30.8±5.1	30.7±3.2
2h	28.6±3.7	31.4±5.0	30.9±4.6	34.2±4.0	32.4±5.3	33.5±5.5
6h	28.8±3.0	33.7±4.4	37.9±3.5	42.6±3.4*	41.5±3.4*	40.6±3.1*
24h	28.9±3.2	34.7±4.0	38.7±1.6*	40.6±3.1*	38.9±2.9*	43.3±1.6°

**p<0.05, °p<0.01 with respect to control cultures.*

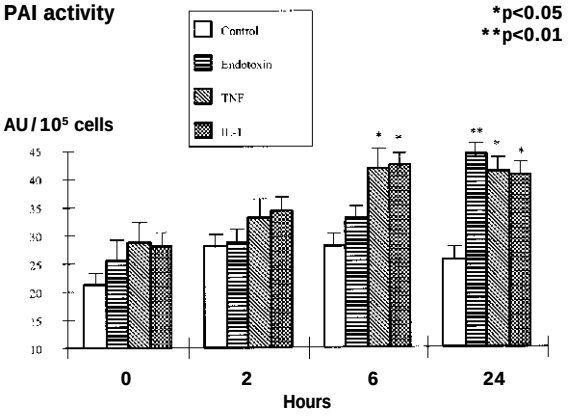

Figure 1. Time course of secreted PAI activity from HUVEC stimulated with 100 ng / mL of endotoxin, TNFα and IL-1α (mean ± SEM values are shown).

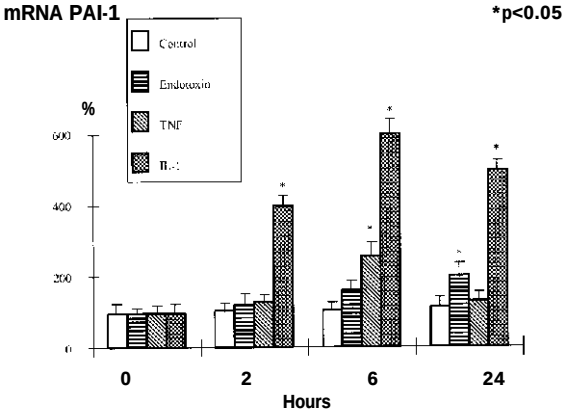

Figure 2. Time course of PAI-1 mRNA induction by endotoxin, TNFα and IL-1α (100 ng / mL) in HUVEC. Each bar represents the data (mean ± SEM values) for PAI-1 mRNA normalized to the level of G3PDH.

Discussion

The fibrinolytic system serves as one of the major endogenous defense mechanisms against intravascular thrombosis.[1] Endothelial cells (ECs) play a critical role in the regulation of intravascular fibrinolytic activity, serving as a source of PAs and their inhibitors, mainly PAI-1.[3-5] EC production of several fibrinolytic components is highly regulated by hormones, growth factors, cytokines and other proteins.[23,24]

The present study was performed to assess the effects of LPS, TNFα and IL-1α on vascular fibrinolysis from HUVEC. These cells were chosen because previous studies have demonstrated that they are more similar to arterial ECs than to ECs isolated from other veins.[25] It is reasonable, therefore, to predict that HUVEC may respond to endotoxin and cytokines in a way similar to arterial ECs.

Endotoxin, TNFα and IL-1α induced a marked impairment of the fibrinolytic potential of HUVEC characterized by a rapid and transient increase of PA activity followed by a significant elevation of PAI-1, a serpin protein which has been implicated in the pathogenesis of thromboembolic disease. A dose dependent increase of PAI-1 activity was observed after HUVEC stimulation with all the agents, although endotoxin and cytokines showed different patterns, suggesting that the modulation of endothelial cell activation is probably regulated through dif-

Table 2. Effect of HUVEC stimulation with different concentrations of endotoxin, TNFα and IL-1α on PA activity measured in the conditioned media. Mean ± SEM are reported.

Endotoxin						
PAI activity (AU/10⁵ cells)	*Control*	*1 ng/mL*	*10 ng/mL*	*100 ng/mL*	*1 µg/mL*	*10 µg/mL*
Basal	0.05±0.01	0.07±0.03	0.05±0.02	0.05±0.02	0.06±0.02	0.05±0.01
2 h	0.06±0.02	0.09±0.01	0.05±0.02	0.05±0.02	0.10±0.01	0.09±0.01
6 h	0.08±0.01	0.11±0.02	0.19±0.04°	0.31±0.11°	0.39±0.12°	0.36±0.13°
24 h	0.17±0.01	0.19±0.02	0.15±0.01	0.16±0.03	0.18±0.02	0.20±0.03
TNFα						
PAI activity (AU/10⁵ cells)	*Control*	*1 ng/mL*	*10 ng/mL*	*100 ng/mL*	*1 µg/mL*	*10 µg/mL*
Basal	0.05±0.10	0.07±0.01	0.06±0.01	0.06±0.01	0.06±0.01	0.06±0.01
2 h	0.06±0.01	0.06±0.01	0.06±0.01	0.10±0.01°	0.09±0.01°	0.09±0.01°
6 h	0.08±0.01	0.09±0.01	0.14±0.02	0.54±0.04°	0.53±0.05°	0.52±0.06°
24 h	0.22±0.02	0.24±0.03	0.22±0.02	0.28±0.02	0.24±0.02	0.22±0.02
IL-1α						
PAI activity (AU/10⁵ cells)	*Control*	*1 ng/mL*	*10 ng/mL*	*100 ng/mL*	*1 µg/mL*	*10 µg/mL*
Basal	0.07±0.01	0.07±0.01	0.07±0.01	0.08±0.01	0.08±0.01	0.09±0.01
2 h	0.06±0.01	0.07±0.01	0.08±0.01	0.25±0.04°	0.20±0.02°	0.17±0.02°
6 h	0.09±0.01	0.09±0.01	0.34±0.01	1.20±0.11°	1.27±0.11°	1.06±0.09°
24 h	0.37±0.06	0.42±0.08	0.31±0.04	0.29±0.01	0.33±0.01	0.36±0.01

**p<0.05, °p<0.01 with respect to control cultures.*

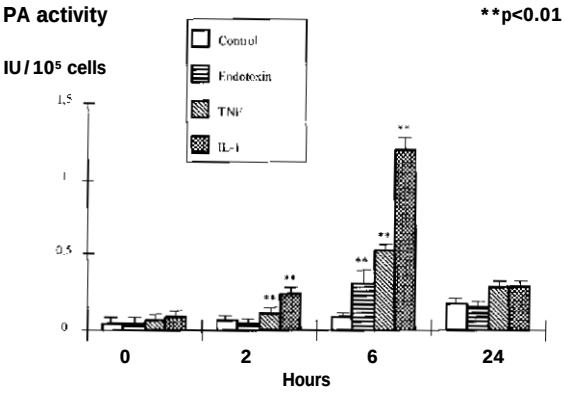

Figure 3. Time course of secreted PA activity from HUVEC stimulated with 100 ng/mL of endotoxin, TNFα and IL-1α (mean ± SEM values are shown).

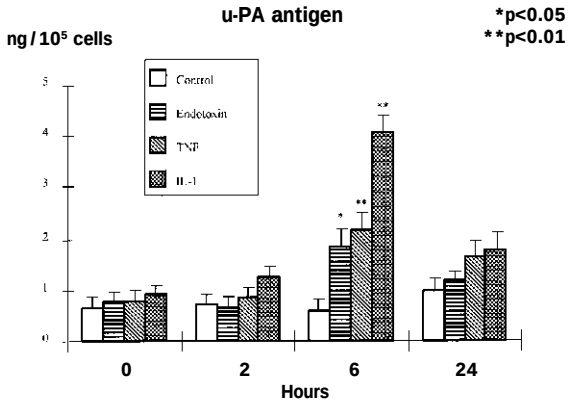

Figure 4. Time course of secreted u-PA antigen from HUVEC stimulated with 100 ng/mL of endotoxin, TNFα and IL-1α (mean ± SEM values are shown).

ferent transduction pathways.[26] PAI activity significantly increased 24 hours after stimulation with endotoxin, whereas an earlier peak (at 6 hours) was observed when HUVEC were stimulated with TNFα and IL-1α indicating that endotoxin and cytokines are potent inducers of PAI secretion from endothelial cells .[11, 27-29] The effect seems to take place at the transcriptional level as shown by the increase in mRNA in cultures stimulated with endotoxin and cytokines, not requiring *de novo* protein synthesis. The observed differences between mRNA and protein secretion would indicate that only a small proportion of total PAI-1 mRNA is expressed as an active protein.[30] A similar pattern of mRNA and protein expression was observed for TNF-α and IL-1 although changes occurred earlier, which is consistent with *in vivo* observations after intravenous administration of these agents to human volunteers.[29,31] Moreover, *in vivo* experiments performed with LPS, TNF and IL-1 have confirmed that the amount of this inhibitor, both locally and systematically, may be of pathological significance for thrombosis and inflammation.[32,33]

PA activity increased in the first hours after stimulation with endotoxin, TNFα and IL-1α to drop at 24 h whereas no effect on t-PA antigen levels was observed in HUVEC stimulated with endotoxin or cytokines. u-PA antigen significantly increased 6 hours after stimulation to drop under control levels at 24 hours. On the other hand, quenching experiments with specific antibodies also showed that the PA activity was mainly due to u-PA, as previously reported.[34] Although there seems to be a discrepancy between the observed PA activity and the time of maximal induction of u-PA activity after stimulation of different cell types, it has been shown that u-PA mRNA level markedly increases at 2 and 6 hours after addition of 500 U/mL TNFα[35] to HUVEC in agreement with the observed u-PA antigen levels in our study. Additional support to explain these findings comes from *in vivo* experiments of TNF infusion to healthy subjects who develop an increase in PA activity, mainly due to u-PA antigen.[32]

In conclusion, endotoxin, TNFα and IL-1α significantly impaired the fibrinolytic potential of HUVEC by inducing an early increase in PA activity, which was counterbalanced by a later enhancement of PAI activity and subsequent fibrinolysis inhibition. Our findings also suggest that the PA activity is related mainly to u-PA rather than to t-PA. The observed changes are in agreement with *in vivo* studies showing that endotoxin and cytokines induce an initial increase of PA which is counterbalanced by the subsequent increase of PAI-1. These induced changes may be of central importance in the pathogenesis of thrombosis related to septic shock and inflammatory reactions.

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JO and JAP were responsible for the conception of the study, interpretation of the data and drafting the manuscript. CC and RM was responsible for data handling, biochemical measurements and interpretation of the results. All the authors contributed to writing the manuscript.

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Disclosures

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Redundant publications: no substantial overlapping with previous papers.

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