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TUMOR NECROSIS FACTOR-α INDUCES THE PHOSPHORYLATION OF 28kDa STRESS PROTEINS IN ENDOTHELIAL CELLS: POSSIBLE ROLE IN PROTECTION AGAINST CYTOTOXICITY?

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SUMMARY. Tumor necrosis factor-α has been shown to rapidly increase the phosphorylation of three 28 kDa proteins in bovine aortic endothelial cells but not in L929 cells. Tumor necrosis factor-α induces the necrosis of the latter cells but not of the former. Arsenite enhanced the phosphorylation of the same 28kDa proteins as tumor necrosis factor-α in the endothelial cells. As stress proteins often play a protective role, we suggest that the phosphorylation of these proteins in endothelial cells may be responsible for the resistance of these cells to tumor necrosis factor-α.

TNF, a cytokine secreted by monocytes in response to endotoxin and other immune and inflammatory stimuli, has pleiotropic effects and acts on different cells types (1). The first described action of TNF was the ability to induce the necrosis of tumors *in vivo* and to kill tumor cells *in vitro* (2). *In vitro*, endothelial cells are resistant to the necrotic action of TNF (3) and they seem to present protective mechanisms against the cytocidal effect of TNF (4). In addition to a cytostatic effect (3), TNF induces pleitropic actions on vascular endothelial cells, which lead to a functional reprogramming of these cells towards a proinflammatory and procoagulant state (4).

ABREVIATIONS

TNF: tumor necrosis factor-α; rhTNF: recombinant human TNF; BAEC: bovine aortic endothelial cells;DMEM: Dulbecco's Modified Eagle's Medium; MEM: Minimum Essential Medium; pi: isoelectric pH; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA: ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic; InsPl: inositol monophosphate; $InsP_2$: inositol bisphosphate; $InsP_3$: inositol trisphosphate.

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The intracellular mediator(s) responsible for the pleiotropic action of TNF on endothelial cells has not yet been identified. The earliest biochemical event detected so far in TNF stimulated endothelial cells is the phosphorylation of a 27kDa band (5). In this paper, we studied the phosphorylation of 28kDa proteins in TNF stimulated BAEC and characterized these substrates as stress proteins.

MATERIALS AND METHODS

BAEC were isolated by collagenase treatment of aortas freshly obtained from the slaughterhouse, as described previously (6,7). They were cultured in a medium consisting of 3:1 DMEM/Ham's F12, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B, supplemented with 20% fetal calf serum. They were used up to the sixth passage.

L929 cells are derived from a mouse fibrosarcoma. They were grown in DMEM supplemented with 10% new-born calf serum, penicillin and streptomycin as above.

For phosphorylation studies, confluent cells were rinsed twice with MEM without phosphate and incubated for 6 h in this same medium containing 200 to 500 µCi/ml [^{32}P]phosphate (Amersham). The total medium phosphate concentration was adjusted to 10 µM with KH_2PO_4. At specified times (1 min. to 4 h) before the end of the labelling, the tested agents were added to the incubation medium. Incubations were stopped by removing medium, rinsing the cells once with NaCl 0.9% (w/v) and adding 200 µl of lysis buffer as previously described (12). The lysates were rapidly frozen in liquid nitrogen. Two-dimensional gel electrophoresis was carried out according to O'Farrell's method (8) modified by Lecocq et al. (9) and Demolle et al. (12). After protein fixation, the gels were dried and exposed to Amersham βMax films at room temperature or to Amersham MP films with an image-intensifying screen (Siemens) at -80°C.

In order to label the phosphoinositides, the cells were incubated in a medium consisting of 3:1 DMEM/Ham's F12 (without inositol), 10% serum and antibiotics, supplemented with myo-(2-^{3}H)inositol (10 µCi/ml) during 48 hours. Then, the cells were washed twice with DMEM, preincubated for 30 min in 1 ml DMEM containing LiCl (10mM) and incubated for periods of 15 sec to 30 min with or without rhTNF. The incubation was stopped by the removal of the medium and addition of perchloric acid. The cellular extract was neutralised with KOH and Hepes at 4°C and submitted to anion-exchange chromatography on Dowex AG1X8 resin, as described (10,13).

Recombinant human TNF (rhTNF) was prepared as described by Tavernier et al. (11).

RESULTS

BAEC incubated for 20 min with rhTNF (2000 U/ml) showed an increase in the phosphorylation of three 28kDa proteins whose approximate pi were 5.5 , 5.8, and 6 respectively (fig. 1). In several experiments, the increase in phosphorylation was most striking for the two more acid variants. This observation was confirmed when the spots were excised out of the dried gel and counted (data not shown). rhTNF also decreased the phosphorylation of a 27kDa protein (pi 6.2). ATP (100 µM; 20 min), as already described (12), induced the phosphorylation of three 28kDa proteins. According to co-migration experiments, these proteins were identical to those phosphorylated in response to rhTNF (data

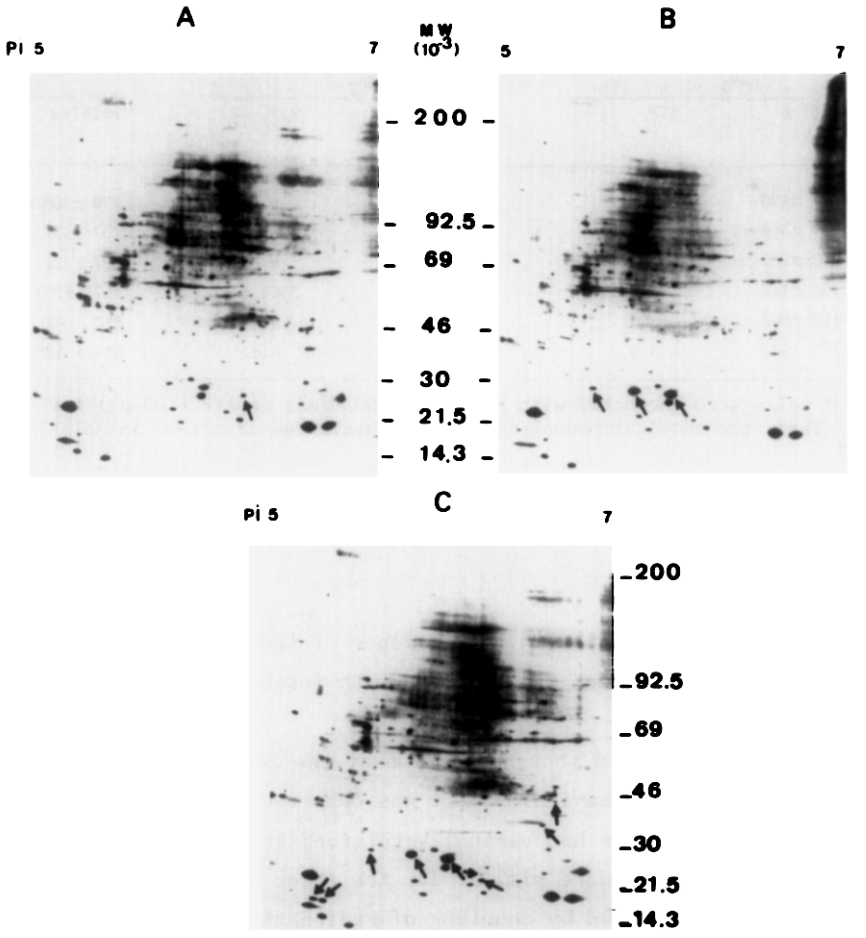

Fig 1. Two dimensional phosphoprotein pattern of BAEC treated with rhTNF and ATP.
BAEC were labeled with [^{32}P] and exposed to rhTNF (2000 U/ml) or ATP (100 μM) for 20 min. Phosphoproteins of control (A), rhTNF (B) or ATP (C) treated cells were analysed by two-dimensional gel electrophoresis. The spots modified by rhTNF or ATP are indicated by arrows on the corresponding gel. The protein whose phosphorylation is decreased in response to ATP and rhTNF is indicated on the control gel. These autoradiographies were from one representative experiment out of four in which each condition was tested in duplicate.
The stimulation of phosphorylation of the three 28kDa spots in response to rhTNF and ATP was confirmed by counting the radioactivity corresponding to the spot: after 20 min, the radioactivity of the central protein in cells incubated with rhTNF was five-fold higher than that of control cells, the phosphorylation of the more basic variant was stimulated three-fold only (data not shown). The radioactivity of the more acidic spot was too weak for accurate counting in the two conditions.

not shown). Additionally, ATP modified the phosphorylation of six other proteins (fig 1) whereas rhTNF had no effect. Moreover, as shown previously (12), this nucleotide induced rapidly (after 30 sec) and transiently (less than 5 min) the phosphorylation of a 95kDa protein (data not shown). Unlike ATP, rhTNF did not modify the intracellular concentration of inositol phosphophates in BAEC (table 1). A similar phosphorylation of the 28kDa proteins was observed

Table 1. Effect of rhTNF-α on the intracellular concentration of insitol phosphates of BAEC; comparison with ATP

Incubat time	$InsP_1$		$InsP_2$		$InsP_3$	
	rhTNF-α	ATP	rhTNF-α	ATP	rhTNF-α	ATP
15 sec	5873 ±406	n.d.	1540 ±169	n.d.	930 ± 60	n.d.
30 sec	5140 ±369	n.d.	1292 ± 65	n.d.	797 ± 37	n.d.
1 min	5842 ±932	n.d.	1372 ± 44	n.d.	837 ± 21	n.d.
5 min	6195 ±112	14137 ±1133	1510 ±271	3448 ± 81	1073 ±231	1870 ± 99
15 min	6199 ±803	n.d.	1710 ±546	n.d.	870 ± 47	n.d.
30 min	5779 ±983	n.d.	1368 ±277	n.d.	874 ± 129	n.d.

The labelled cells were incubated with rhTNF-α (2000 U/ml) or ATP (100 μM) for the time specified. Then, the inositol phosphates were separated as described in MATERIALS AND METHODS.
The results for the control conditions were 5803 ±553 for $InsP_1$, 1446 ±162, for $InsP_2$ and 886 ±16 for $InsP_3$. These results did not change significantly with the time of incubation.

when the cells were incubated in the presence of the calcium ionophore A23187 (100 nM) (12), but no effect was observed with forskolin (10 μM) (data not shown).

The rhTNF induced phosphorylation of the 28kDa proteins was not observed before 3 min, a maximal effect was observed after 20 min. After 4 hours, the phosphorylation had virtually returned to control level (fig 2). The phosphorylation kinetics were similar for the three 28 kDa proteins. These observations were confirmed by counting of radioactivity spots (data not shown).

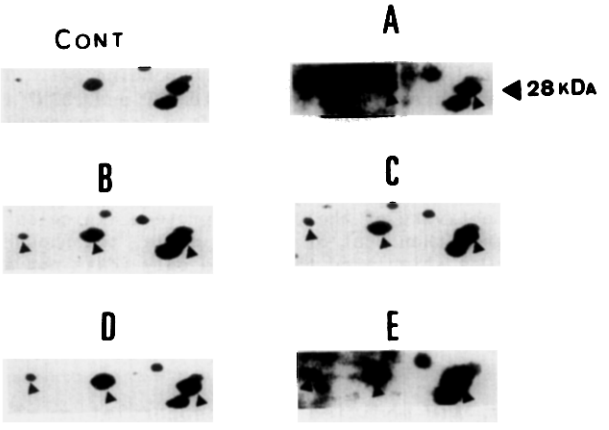

Fig 2. Kinetic of action of rhTNF on the phosphorylation of the 28kDa proteins of BAEC.
The [^{32}P]phosphate labelled cells were incubated with rhTNF (2000 U/ml) for 1 min (A), 3 min (B), 5 min (C), 20 min (D). For the 4 hour incubation (E), the cytokine was added after 2 hours of labelling. These autoradiographies were from one representative experiment out of two in which each condition was tested in duplicate.

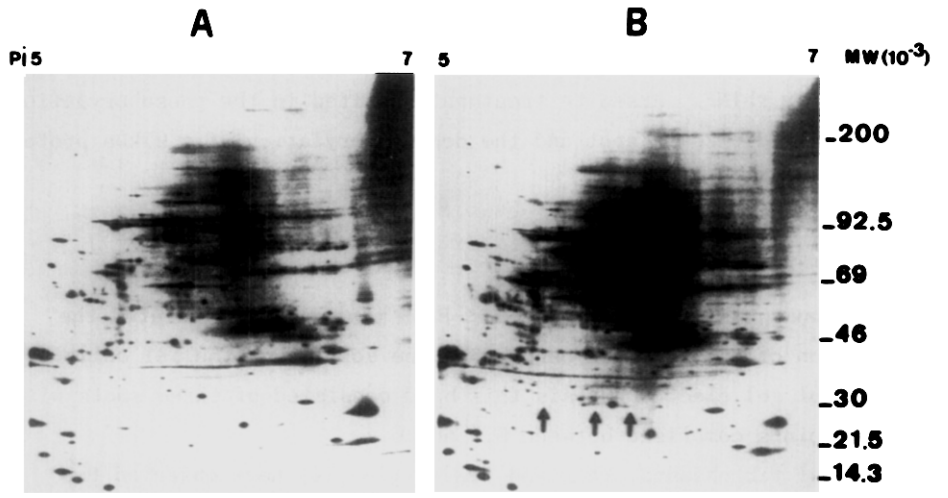

Fig 3. Two dimensional phosphoprotein pattern of L929 cells treated with rhTNF. The [^{32}P]phosphate labelled cells were incubated for 20 min without (A) or with rhTNF (2000 U/ml) (B). The 28kDa proteins localisation (detected by co-migration of BAEC and L929 proteins) is indicated by arrows. These autoradiographies were from one representative experiment out of two in which each condition was tested in duplicate.

L929 cells are sensitive to the cytotoxic effect of TNF. Incubation with rhTNF during 20 min did not elicit any modification in the phosphorylation of 28kDa or other proteins (fig 3).

After an incubation of BAEC in a medium containing 100 µM sodium arsenite, three 28kDa proteins presented a obvious increase in phosphorylation (fig 4). Co-migration on the same gel of proteins from arsenite and rhTNF treated cell samples showed that these proteins had identical molecular weights and

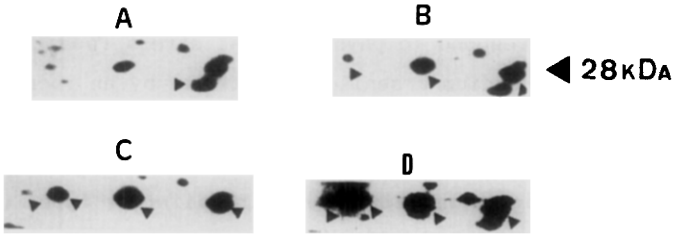

Fig 4. Effect of arsenite on the phosphorylation of the 28kDa proteins in BAEC. A: control; B: rhTNF treated cells (20 min); C: arsenite treated cells (4 hours) and D: co-migration.
The cells were incubated for 6 hours in a medium containing [^{32}P]phosphate. In the same medium arsenite (100 µM) was added. Arsenite (100 µM) was added in this medium 4 hours before the end of the labelling. The 27kDa dephosphorylated after arsenite treatement is indicated by an arrow on the control autoradiograph. For rhTNF treated cells, the procedure was the same as exposed previously. For the co-migration experiment, a same amount of radioactivity of the arsenite and rhTNF treated samples was applied on a unique gel. These autoradiographies were from one representative experiment out of three in which each condition was tested in duplicate.

isoelectric points suggesting that they were the same proteins. The increases in phosphorylation induced by arsenite were significantly more important than those induced by rhTNF. Arsenite treatment resulted in the phosphorylation of an additional acidic 28kDa spot and the dephosphorylation of a 27kDa protein (fig 4).

DISCUSSION

We have previously shown by SDS-PAGE that rhTNF stimulated the phosphorylation of one 27-28kDa band in bovine aortic endothelial cells (5). In two dimensional gel electrophoresis this band consisted of three spots with isoelectric points comprised between 5.5 and 6.
In human dermal fibroblasts, Kaur and Saklatvala (14) have observed the phosphorylation of a similar triad of proteins in response to TNF and the kinetics of phosphorylation were comparable to those seen in BAEC. Schulsse et al. have reported that TNF stimulated the phosphorylation of a 26kDa protein in the human monoblastoid cell line, U937. However, the stimulation had an earlier onset (20 sec) and only one variant was detected (15).

In BAEC, phosphorylation of the three 28kDa proteins is also stimulated by ATP and bradykinin (12). These agonists are known to induce the accumulation of $InsP_3$ (13,16,17) and subsequently of cytosolic Ca^{2+} in these cells (13,16). As the ionophore A23187 causes the same effect, Demolle et al. have suggested that this phosphorylation is catalysed by a calmodulin-dependent kinase (12). rhTNF does not modify the intracellular concentration of $InsP_3$, nor the cytosolic level of Ca^{2+} (data not shown) in BAEC. This observation suggests that, contrary to ATP, the stimulation of phosphorylation induced by rhTNF did not involve a Ca^{2+}-dependent kinase. This is supported by the fact that rhTNF did not reproduce the other effects of ATP and A23187 on the phosphorylation pattern in BAEC.

Mammalian cells respond to physiological stress (heat shock, incubation in presence of sodium arsenite or cadmium) by an increase in the synthesis and/or the phosphorylation of different proteins called "stress proteins" (18,19). Among these proteins, there is the family of 28kDa proteins composed of four related isoforms presenting, in different types of cells, a similar electrophoretic pattern (19-22). Our experiments showed that sodium arsenite increased the phosphorylation of four 28kDa proteins in BAEC. By co-migration experiments, we could establish that the three more acidic variants phosphorylated in stress conditions are the same as those phosphorylated in response to rhTNF. These observations suggest that rhTNF stimulated the phosphorylation of three 28kDa stress proteins in BAEC.

Different studies have shown the phosphorylation of similar proteins in non-stress conditions,suggesting that it may play a central role in cellular

responses to many stimuli independently of physiological stress (19,21,23-25). The phosphorylation stimulated by rhTNF might thus also be involved in the reprogrammation of BAEC towards a proinflammatory and procoagulant state (4). The protective role of the 28kDa proteins in stress conditions seems well established, principally in thermoresistance (22), although the biochemical fonction of these proteins remains to be discovered. Chrétien and Landry (22) suggested that the response to a physiological stress involves, in sequence: 1) rapid phosphorylation giving an immediate protection and 2) induction of stress proteins synthesis that peaks after a few hours, increasing this capacity of the mechanism. Indeed, agents such as A23187, serum and EGTA which stimulate the phosphorylation of 28kDa stress proteins without increasing their synthesis, partially protect the cells against hyperthermic killing, whereas arsenite which induces both phosphorylation and synthesis, gives a more efficient and durable protection against hyperthermic stress.

Cell insensitive to the necrotic action of TNF, as the endothelials cells (3), seem to present an active mechanism of protection against the cytokine (26). Extending to TNF action the concept developed for physiological stress, we suggest that the phosphorylation of the 28kDa proteins stimulated by rhTNF may perhaps play a role in the protective mechanism of BAEC against the necrotic action of TNF. This hypothesis is supported by the fact that this phosphorylation is absent in L929 cells which are sensitive to the cytotoxic action of TNF. Further experiments are in progress in order to test this hypothesis.

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