Hyperosmolarity stimulates prostaglandin synthesis and cyclooxygenase-2 expression in activated rat liver macrophages

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The effect of aniso-osmotic exposure on the level of inducible cyclooxygenase (Cox-2) and on prostanoid synthesis was studied in cultured rat liver macrophages (Kupffer cells). In lipopolysaccharide (LPS)- or phorbol 12-myristate 13-acetate-stimulated Kupffer cells, hyperosmotic (355 mosmol/l) exposure, due to addition of NaCl or impermeant sugars, markedly increased prostaglandin (PG) E_2, D_2 and thromboxane B_2 synthesis in a time- and osmolarity-dependent manner. Increased prostanoid production was observed about 8 h after exposure to LPS in hyperosmotic medium compared to Kupffer cells treated with LPS under normotonic (305 mosmol/l) conditions. A similar stimulatory effect of hyperosmolarity on PGE_2 production was also seen when arachidonate was added exogenously. Hyperosmotic stimulation of PGE_2 production was accompanied by a strong induction of Cox-2 mRNA levels and an increase in immunoreactive Cox-2, whereas the levels of immunoreactive phospholipase A_2 and cyclooxygenase-1 did not change significantly. Dexamethasone, indomethacin and the selective Cox-2 inhibitor, NS-398, abolished the hypertonicity-induced stimulation of PGE_2 formation; dexamethasone also prevented the increase in Cox-2 mRNA and protein. The increase of immunoreactive Cox-2 lasted for about 24 h and was also blocked by actinomycin D or cycloheximide, but not by brefeldin A. Tunicamycin or treatment with endoglucosidase H reduced the molecular mass of hypertonicity-induced Cox-2 by 5 kDa. Tunicamycin treatment also suppressed the hypertonicity-induced stimulation of PGE_2 production. The hyperosmolarity/LPS-induced stimulation of prostaglandin formation was partly sensitive to protein kinase C inhibition but was not accompanied by an increase in the cytosolic free Ca^{2+} concentration. The data suggest that osmolarity may be a critical factor in the regulation of Cox-2 expression and prostanoid production in activated rat liver macrophages.

INTRODUCTION

Kupffer cells are the major producers of eicosanoids, such as prostaglandin (PG) E_2, D_2 and thromboxane A_2, in liver [1]. The pattern of prostanoid release by Kupffer cells depends on the type of their activation. Inflammatory agents like lipopolysaccharide (LPS), viruses or cytokines induce PGE_2 preferentially, while phagocytosis, phorbol ester or elevations of intracellular Ca^{2+} make PGD_2 the major eicosanoid released [1–10]. Eicosanoid production by liver macrophages plays a major role in the pathogenesis of septic shock and may contribute to liver cell damage under these conditions [11,12]. Modulation of prostaglandin production occurs at the level of (i) arachidonate liberation by phospholipases A_2 (PLA_2) and C [13] and (ii) conversion of the released arachidonate into prostaglandins by cyclooxygenase, which is synonymous with prostaglandin H synthase. Cyclooxygenase is a bifunctional enzyme with both oxygenase and peroxidase activity [14] and is present in two isoforms [15–17]. One isoform (Cox-1) is constitutively expressed, whereas the inducible isoform (Cox-2) appears in macrophages in response to proinflammatory stimuli [4,7,10,18].

Severe infectious diarrhoea may not only be accompanied by an increased endotoxin load to the liver but also by plasma hyperosmolarity and hypernatremia due to extensive fluid losses. However, nothing is known about the regulation of Kupffer cell function by ambient osmolarity, although in other cells types, such as liver parenchymal cells, aniso-osmotic cell volume changes have recently been recognized as important determinants of metabolic cell function and gene expression [19–21]. Thus, we studied the effect of aniso-osmolarity on prostaglandin synthesis in rat Kupffer cells. It was observed that hyperosmotic exposure markedly enhances prostaglandin synthesis in LPS-activated rat Kupffer cells due to increased synthesis of Cox-2.

MATERIALS AND METHODS

Materials

LPS (from *Salmonella minnesota* R595) was provided by Dr. C. Galanos (Max-Planck-Institut für Immunbiologie, Freiburg, Germany). The protein kinase C (PKC) inhibitor, Gö 6850 [22], was provided by Dr. A. Schächtele (Gödecke Freiburg, Germany). Verapamil, tunicamycin, brefeldin A, fura 2 acetomethoxyester, arachidonic acid and endoglucosidase H were from Sigma (Deisenhofen, Germany). α-D-Raffinose was from Serva (Heidelberg, Germany). AACOCF3, which is an arachidonic acid derivative in which the -OH is replaced by $-CF_3$ [23], was from Biomol (Hamburg, Germany). Pronase and collagenase

Abbreviations used: LPS, lipopolysaccharide; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate; PGE_2, prostaglandin E_2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PLA_2, phospholipase A_2; Cox-1, constitutive cyclooxygenase; Cox-2, inducible cyclooxygenase; PKC, protein kinase C; LDH, lactate dehydrogenase; MAP, mitogen-activated protein; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

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were from Boehringer Mannheim (Mannheim, Germany). Culture medium RPMI 1640 (without Phenol Red) and fetal calf serum (FCS) were from Biochrom (Berlin, Germany), phorbol 12-myristate 13-acetate (PMA) and the oligonucleotide-labelling kit were from Pharmacia (Freiburg, Germany). Guanidine thiocyanate and sodium lauroylsarcosinate were from Fluka (Karlsruhe, Germany). [α-^{32}P]dCTP (3000 Ci/mmol) and Hybond-N nylon membranes were purchased from Amersham Buchler (Braunschweig, Germany). NS-398, a specific inhibitor of Cox-2 [24] and the cyclooxygenase (Cox-1 and Cox-2) cDNA probes were from Cayman Chemical Company (Ann Arbor, MI, U.S.A.) and the 1.0 kb cDNA fragment for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used for standardization was from Clontech (Palo Alto, CA, U.S.A.).

Isolation and culture of Kupffer cells

Kupffer cells from male Wistar rats of 300–400 g body weight (Interfauna, Tuttlingen, Germany) were isolated by collagenase–Pronase perfusion and separated by a single Nycodenz gradient and centrifugal elutriation according to [25]. Cells were cultured in RPMI 1640 medium supplemented with 20% heat-inactivated FCS for 48 h. The experiments were performed during the following 24 h using Krebs–Henseleit hydrogen carbonate buffer (pH 7.4) containing 10 mM glucose and 2.5% FCS. The osmolarity was varied by changing the NaCl concentration. The viability of Kupffer cells was more than 95%, as assessed by Trypan Blue exclusion. Viability of the incubations was routinely tested by lactate dehydrogenase (LDH) release at the end of the incubations.

Determination of PGE_2

Kupffer cell supernatants were assayed for PGE_2 by competitive binding radioimmunoassay using ^{3}H-labelled PGE_2 (Amersham, Braunschweig, Germany) and a specific antiserum to PGE_2 (Sigma, Deisenhofen, Germany). Prostanoids were also analysed by HPLC as described earlier [25]. In brief, 10^7 Kupffer cells were prelabelled with [^{3}H]arachidonic acid (1 μCi/ml) for 24 h. After washing, the Kupffer cells were incubated in the label-free experimental medium for 24 h. Thereafter, the labelled prostaglandins were extracted and separated on HPLC according to [25].

Western blotting

Kupffer cells were washed with PBS and were lysed in 100 mM NaCl containing 10 mM Tris/HCl (pH 7.3), 2 mM EDTA, 0.5% deoxycholate, 1% Nonidet P.40, 10 mM $MgCl_2$, 1 mM PMSF and 10 μg of aprotinin/ml for 10 min on ice. Lysates containig 30 μg of protein were mixed with an equal volume of Laemmli sample buffer and denatured by boiling for 5 min. After SDS/PAGE (10% gel) and electrophoretic transfer, the nitrocellulose filters were blocked using 3% defatted dried milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h. Filters were incubated overnight with a specific antibody to Cox-2 (Cayman Chemicals, Ann Arbor, MI, U.S.A.) used at a dilution of 1:1000 or a monoclonal anti-Cox-1 antibody (Cascade Biochem Ltd) used at a dilution of 1:200 or anti-pancreas PLA_2 (UBI, Lake Placid, NY, U.S.A.), which detects cellular PLA_2 [26], used at a dilution of 1:1000. After washing in TBS-T, the filters were incubated with horseradish peroxidase-conjugated anti-rabbit antibody or anti-mouse antibody, again washed four times in TBS-T and exposed to enhanced chemiluminescence reagents for 1 min; blots were exposed to Kodak XAR-5 film for 1–5 min. In some experiments, the cell lysates containing 30 μg of protein were incubated for 16 h at 37 °C in the presence of 0.002 unit of endoglucosidase H [27]. Then, the samples were boiled for 5 min and subjected to electrophoresis using SDS/PAGE (10%) and Western blot analysis was performed as described above.

Northern blot analysis

Total RNA from near-confluent culture plates of Kupffer cells was isolated by using guanidine thiocyanate solution as described in [28]. RNA samples were electrophoresed in 0.8% agarose/3% formaldehyde and then blotted onto Hybond-N nylon membranes with 20 × SSC (3 M NaCl/0.3 M sodium citrate). After brief rinsing with water and UV-crosslinking (Hoefer UV-crosslinker 500), the membranes were observed under UV illumination to determine RNA integrity and location of the 28 S and 18 S rRNA bands. Blots were then subjected to a 3 h prehybridization at 43 °C in 50% de-ionized formamide in sodium phosphate buffer (0.25 M; pH 7.2) containing 0.25 M NaCl, 1 mM EDTA, 100 mg/ml salmon sperm DNA and 7% SDS. Hybridization was carried out in the same solution with approx. 10^6 c.p.m./ml [α-^{32}P]dCTP-labelled random primed Cox-1 or Cox-2 and GAPDH cDNA probes. Membranes were washed three times in 2 × SSC/0.1% SDS for 10 min, twice in sodium phosphate buffer (25 mM; pH 7.2)/EDTA (1 mM)/0.1% SDS and twice in sodium phosphate buffer (25 mM; pH 7.2)/EDTA (1 mM)/1% SDS. Blots were then exposed to Kodak AR X-omat film at −70 °C with intensifying screens. Suitably exposed autoradiograms were then analysed with densitometry scanning (PDI, New York, NY, U.S.A.) to determine the absorbances of the mRNA levels for Cox and GAPDH. Relative Cox mRNA levels were determined by standarization to the absorption of GAPDH mRNA.

Determination of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

Rat Kupffer cells were cultivated on coverslips and were pre-incubated with Krebs–Henseleit medium (115 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 5.9 mmol/l KCl, 1.18 mmol/l $MgCl_2$, 1.2 mmol/l NaH_2PO_4, 1.2 mmol/l Na_2SO_4, 1.25 mmol/l $CaCl_2$ and 6 mmol/l glucose), containing 5 μmol/l fluorescence chelator fura 2 acetomethoxyester and 0.02% pluronic F-127, for 30 min at 30 °C and 5% CO_2. For fluorescence recording, the coverslips were continuously superfused at a rate of 15 ml/min with Krebs–Henseleit buffer at 37 °C, equilibrated with O_2/CO_2 (95/5; v/v), resulting in a pH of 7.4. Measurement of $[Ca^{2+}]_i$ was performed at the single cell level as described in [29].

Statistics

Values are expressed as means ± S.E.M (n = number of Kupffer cell preparations). For statistical analyses the non-parametric H-test of Kruskal and Wallis and the U-test of Wilcoxon, Mann and Whitney were used as indicated. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of aniso-osmotic exposure on prostanoid production in activated rat Kupffer cells

PGE_2 production by LPS-stimulated rat Kupffer cells after exposure to aniso-osmotic media for 24 h (Figure 1a) revealed that an increase in the osmolarity of the medium above 305 mosmol/l, due to addition to NaCl, was able to strongly

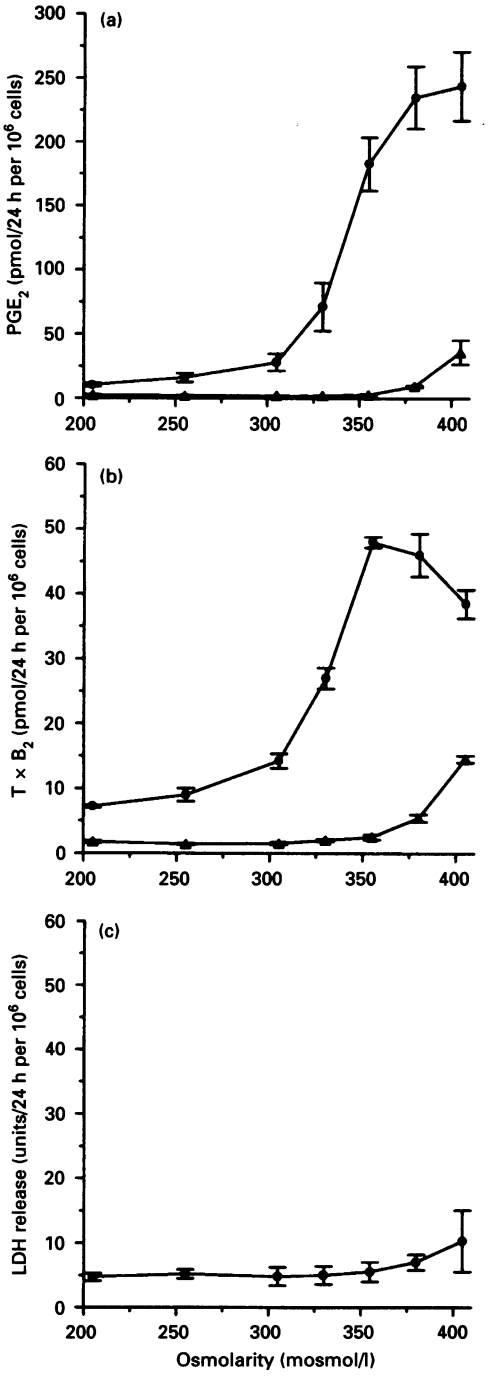

Figure 1 Hyperosmolarity increases LPS-elicited PGE_2 (a) and thromboxane B_2 (b) synthesis by rat Kupffer cells

Kupffer cells were incubated in Krebs–Henseleit buffer of different osmolarity as indicated without LPS (▲) or with LPS (●) (1 μg/ml) for 24 h. PGE_2 (**a**) and thromboxane B_2 (TxB_2) (**b**) accumulating over the 24 h test periods were measured by radioimmunoassay. (**c**) LDH activity found in the supernatant of LPS-treated Kupffer cells after a 24 h incubation in media with different osmolarity. Data are given as the means ± S.E.M. of at least four independent cell preparations. The effect of osmolarity on PGE_2 formation was statistically significant (The H-test of Kruskal and Wallis: $P < 0.01$ in the control group and $P < 0.001$ in the LPS-stimulated cells). The H-test also revealed a statistically significant effect of osmolarity on TxB_2 formation in LPS-treated cells ($P < 0.01$) and in controls ($P < 0.001$). Osmolarity had no significant effect on LDH release.

potentiate LPS-stimulated PGE_2 production by Kupffer cells. Similar observations were made with respect to thromboxane B_2 formation (Figure 1b). On the other hand, hypo-osmotic exposure (255 mosmol/l), slightly, but statistically significantly ($P < 0.05$), diminished PGE_2 and thromboxane B_2 formation in LPS-treated cells. Also, in the absence of LPS, formation of PGE_2 and thromboxane B_2 was dependent upon the osmolarity of the medium; the effect was small but statistically significant ($P < 0.01$ and $P < 0.001$ respectively). As shown in Figure 1(c), cell viability, as assessed from the activity of LDH in the supernatant at the end of the 24 h incubations, was not significantly affected by osmolarity when in the range 205–405 mosmol/l.

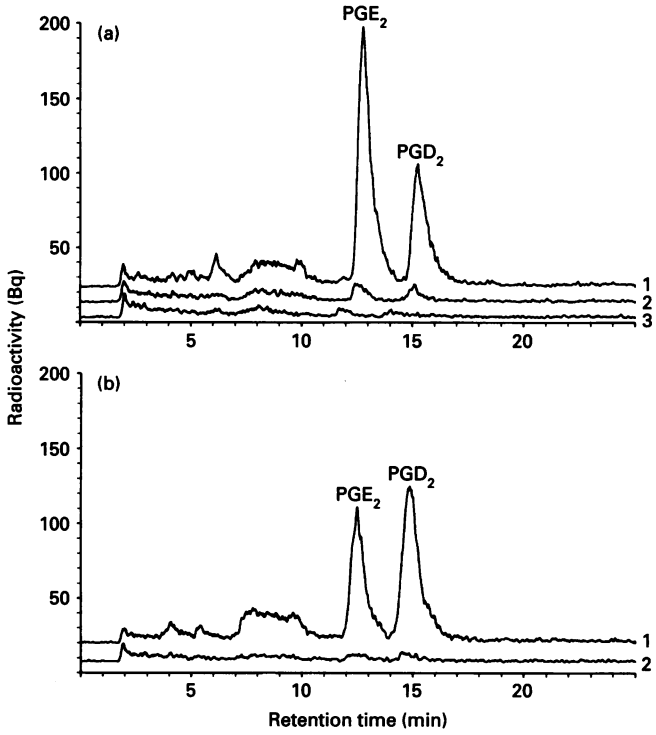

Figure 2 Effects of hyperosmotic (355 mosmol/l) and hypo-osmotic (255 mosmol/l) exposure on the production of PGs in LPS-treated rat Kupffer cells

Kupffer cells were prelabelled with [^{3}H]arachidonate as described in the Materials and methods section. Then they were exposed to media with osmolarities of 255 (trace 3), 305 (trace 2) and 355 mosmol/l (trace 1) in the presence of LPS (1 μg/ml) for 24 h and the supernatant was subjected to HPLC analysis. Osmolarity changes were performed by addition or removal of 25 mmol/l NaCl to/from the normo-osmotic (305 mosmol/l) Krebs–Henseleit medium (**a**). In (**b**), hyperosmotic conditions were instituted by addition of 50 mM raffinose (trace 1). Trace 2 shows the result for cells exposed to normo-osmotic (305 mosmol/l) medium. No PGs were detectable when the incubations were carried out in the presence of indomethacin (results not shown). Representative data obtained by HPLC analysis are shown; the experiments were reproduced at least three times.

Stimulation of prostaglandin production in LPS-activated Kupffer cells by hyperosmotic exposure was also shown by chromatographic analysis of labelled prostaglandins produced by cells in which the membrane phospholipids were prelabelled by prior incubation with [^{3}H]arachidonate. Analysis by reverse-phase HPLC (Figure 2) revealed PGE_2 and PGD_2 as the major prostaglandins produced by rat Kupffer cells. In the presence of LPS the cells in hyperosmotic media synthesized more PGE_2 and PGD_2 than in normo-osmotic (305 mosmol/l) or hypo-osmotic (255 mosmol/l) media (Figure 2a). Stimulation of prostaglandin synthesis by hyperosmolarity was also observed when hyperosmotic conditions (355 mosmol/l) were created by addition of 50 mmol/l raffinose (Figure 2b).

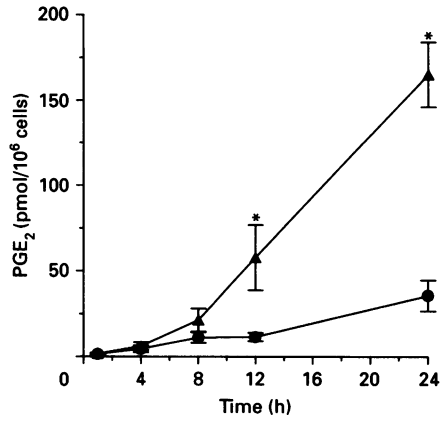

Figure 3 Time-course of LPS-elicited PGE_2 release in normo-osmotic (●) and hyperosmotic (▲) media

Kupffer cells were preincubated with LPS (1 μg/ml) for 12 h; then the cells were exposed to LPS in hyperosmotic (355 mosmol/l) (▲) or normo-osmotic (305 mosmol/l) (●) media for the indicated time periods and PGE_2 was measured by radioimmunoassay. Hyperosmotic conditions were achieved by addition of 25 mmol/l NaCl to the normo-osmotic (305 mosmol/l) Krebs–Henseleit buffer. The results represent means ± S.E.M. of four independent experiments. * Significantly different from 305 mosmol/l (U-test, $P < 0.05$).

Table 1 Effect of hyperosmotic exposure on PGE_2 synthesis by Kupffer cells stimulated with LPS, PMA or A23187

Kupffer cells were incubated in normo-osmotic (305 mosmol/l) or hyperosmotic (355 mosmol/l; due to addition of 25 mmol/l NaCl)) Krebs–Henseleit buffer respectively, and exposed to either PMA (0.1 μmol/l), the Ca^{2+} ionophore A23187 (1 μmol/l) or LPS (1 μg/ml) for 24 h. Results are given as means ± S.E.M. ($n = 4$). * Significantly different from values at 305 mosmol/l (U-test, $P < 0.05$).

	PGE_2 (pmol/24 h per 10^6 cells)	
	Osmolarity (mosmol/l)	
Effector	305	355
Control	2.6 ± 0.2	7.6 ± 1.2*
LPS	29.3 ± 3.6	176 ± 25*
PMA	48.0 ± 13.1	192 ± 7.5*
A23187	193 ± 47	275 ± 27

As shown in Figure 3, the hyperosmolarity-induced stimulation of PGE_2 production occurred after a lag phase of about 8 h (Figure 3). A similar lag phase was observed when the formation of thromboxane B_2 was studied (results not shown).

Short-term exposure to phorbol esters is known to activate PKC and to stimulate prostaglandin formation by Kupffer cells [2,3,6,8]. Also prolonged (24 h) exposure to PMA, which down-regulates PKC but stimulates PGE_2 synthase activity, increases PGE_2 formation [6]. As shown in Table 1, the release of PGE_2 from Kupffer cells in response to a 24 h exposure to PMA was markedly enhanced following hyperosmotic cell shrinkage. On the other hand, PGE_2 release under the influence of the Ca^{2+} ionophore A23187 was already very high and was not significantly stimulated by hyperosmotic conditions (Table 1).

Table 2 Effect of various inhibitors on PGE_2 synthesis elicited by LPS in hypertonic media

Kupffer cells were incubated with LPS (1 μg/ml) in hypertonic (355 mosmol/l) media (= control) supplemented with the indicated substances for 24 h. The values are means ± S.E.M. of four independent experiments. * Significantly different from the control (U-test, $P < 0.05$). LDH, lactate dehydrogenase.

Effector	PGE_2 (pmol/10^6 cells)	Inhibition (%)	LDH leakage (units/10^6 cells)
Control	162 ± 16	0	5.6 ± 0.7
Indomethacin, 10 μM	< 0.5*	99	6.0 ± 0.4
NS-398, 10 μM	3 ± 1*	98	5.1 ± 0.3
AACOF3, 100 nM	41 ± 6*	75	7.6 ± 0.7
Verapamil, 20 μM	128 ± 14	21	6.1 ± 1.0
Dexamethasone, 1 μM	3 ± 1*	98	4.9 ± 0.5
Gö 6850, 1 μM	63 ± 12*	61	6.1 ± 1.0

Characterization of the hyperosmolarity-induced stimulation of PGE_2 formation

As shown in Table 2, the hypertonicity-induced stimulation of PGE_2 synthesis was significantly inhibited in the presence of dexamethasone or $AACOCF_3$ (an arachidonic acid derivative with substitution of the hydroxyl moiety by $-CF_3$ [23]), i.e. an inhibitor of PLA_2. Indomethacin, an inhibitor of cyclo-oxygenases, abolished the hypertonicity-induced stimulation of PGE_2 production and decreased PGE_2 production to levels close to the detection limit (< 0.5 pmol/24 h per 10^6 cells). Also NS-398, a selective inhibitor of Cox-2 [24], strongly inhibited the hypertonicity/LPS-induced increase in PGE_2 formation. In these experiments NS-398 was employed at a concentration of 10 μmol/l, i.e. a concentration recently reported to be without inhibitory action on the Cox-1 isoenzyme [24]. Such a selectivity may also be reflected by the present findings in that (i) NS-398 had no significant effect on PGE_2 formation in unstimulated Kupffer cells (results not shown) and (ii) NS-398 reduced PGE_2 formation to this 'unstimulated' level in Kupffer cells which were exposed to LPS and hyperosmotic medium, whereas indomethacin lowered PGE_2 production close to the detection limit. In normo-osmotically and hyperosmotically (355 mosmol/l) exposed LPS-treated Kupffer cells, PGE_2 formation was 40 ± 5 and 162 ± 16 pmol/24 h per 10^6 cells respectively. These rates were decreased by NS-398 to 2.5 ± 0.6 and 2.8 ± 0.6 pmol/24 h per 10^6 cells respectively, i.e. similar to the rate of PGE_2 formation of 2.6 ± 0.2 pmol/24 h per 10^6 cells found in normo-osmotically exposed unstimulated cells. Verapamil did not affect the hypertonicity-induced stimulation of PGE_2 formation. Gö 6850, an inhibitor of most PKC isoforms [22], had no significant effect on the LPS-stimulation of PGE_2 formation in normotonic media (results not shown), but markedly attenuated the stimulation in response to hyperosmotic exposure (Table 2). This may suggest an involvement of PKC in the hypertonicity-induced stimulation of PGE_2 synthesis.

Effect of exogenously added arachidonate

Prostaglandin formation in macrophages requires the activities of both, PLA_2 and cyclooxygenase(s) and both enzymes have been suggested to exert control on the pathway of PG formation from phospholipids [1,14]. Therefore the effect of exogenously added arachidonate (2 and 10 μmol/l) on PGE_2 formation was examined. It should be mentioned that an arachidonate con-

Table 3 Effect of exogenously added arachidonate on the PGE_2 synthesis in Kupffer cells

Kupffer cells were incubated with or without arachidonic acid (AA) in normotonic (305 mosmol/l) or hypertonic (355 mosmol/l) media with or without LPS (1 μg/ml) for 24 h. * Significantly different from corresponding experiments at 305 mosmol/l (U-test, $P < 0.05$).

Medium osmolarity (mosmol/l)	PGE_2 (pmol/24 h per 10^6 cells) Without AA ($n = 8$)	AA (2 μmol/l) ($n = 5$)	AA (10 μmol/l) ($n = 3$)
305	3±0	4±1	9±2
355	6±1*	16±3*	25±4*
305+LPS	40±5	95±12	133±20
355+LPS	149±9*	236±11*	298±31*

centration of 10 μmol/l does not saturate cyclooxygenase [6], however, cell viability was impaired at higher arachidonate concentrations in LPS/hyperosmotic incubations. Exposure of the cells to LPS, arachidonate (10 μmol/l) and hyperosmolarity (355 mosmol/l) had no effect on cell viability (results not shown). As shown in Table 3, addition of arachidonate (10 μmol/l) stimulated PGE_2 formation, regardless of whether LPS was present or not or whether the cells were incubated in normo-osmotic or hyperosmotic media. These findings may be taken as an indication that PLA_2 exerts control on the formation of PGE_2 from membrane-bound arachidonate. However, in the absence or presence of LPS, hyperosmotically exposed cells produced more PGE_2 in the presence of exogenous arachidonate than did normo-osmotically exposed cells. This finding suggests that hyperosmolarity might increase the activity of cyclooxygenase(s). In line with the control strength theory [30], which predicts that flux control is shared between various enzymes in a metabolic pathway, the findings in Table 2 would be compatible with a control of PGE_2 formation at both sites, i.e. PLA_2 and cyclooxygenase.

Induction of Cox-2 in Kupffer cells by hyperosmotic exposure

LPS treatment is known to induce the Cox-2 isoform of cyclooxygenase [4,7,10], and glucocorticoids have previously been shown to inhibit expression of Cox-2 mRNA in LPS-PMA- and cytokine-stimulated cells [7,31]. As shown in Figure 4, addition of LPS to normotonically exposed rat Kupffer cells induces the Cox-2 protein within 1 h, whereas in normo-osmotic control incubations (LPS absent) Cox-2 is not detectable by Western blotting over an incubation period of 24 h. Hyperosmotic exposure, which up to 4 h had little effect on the LPS-

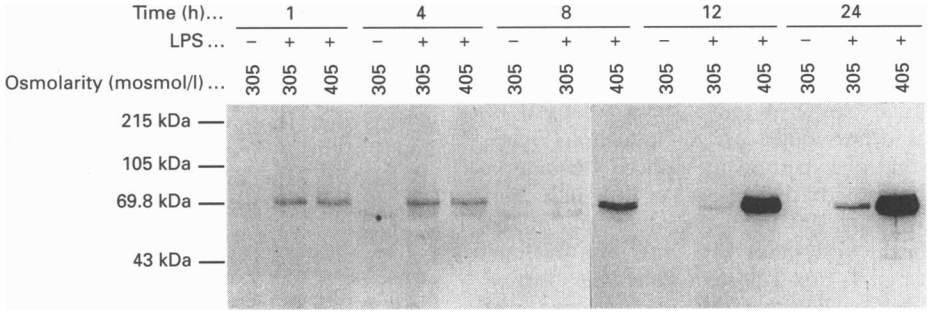

Figure 4 Time-course of induction of Cox-2 protein in Kupffer cells after stimulation with LPS and/or hyperosmotic environment

Kupffer cells were incubated in normo-osmotic control medium or with LPS (1 μg/ml) and hyperosmotic (405 mosmol/l) or normo-osmotic (305 mosmol/l) medium for the indicated time-periods. Western blot analysis was performed with an antibody specific for Cox-2 as described in the Materials and methods section.

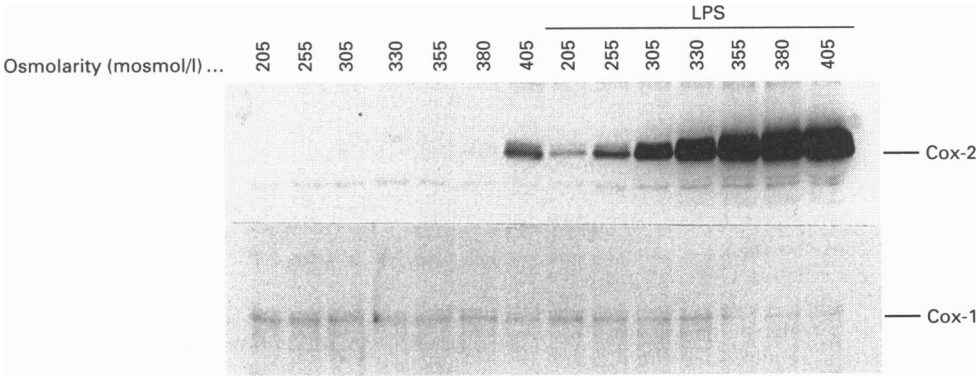

Figure 5 Osmolarity-dependent accumulation of Cox-2, but not Cox-1, protein in LPS-stimulated Kupffer cells

Kupffer cells were incubated for 24 h without or with LPS (1 μg/ml) in media with different osmolarities. Osmolarity changes were performed by addition or removal of corresponding amounts of NaCl to/from the normo-osmotic Krebs–Henseleit medium (305 mosmol/l). Western blot analysis was performed with antibodies specific for Cox-1 and Cox-2 as described in the Materials and methods section.

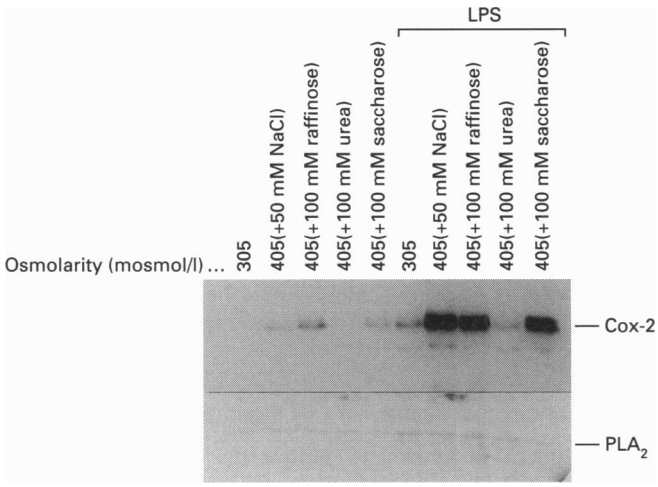

Figure 6 Effect of hyperosmolarity on Cox-2 and PLA_2 in LPS-treated and untreated Kupffer cells

Kupffer cells were incubated for 24 h without or with LPS (1 μg/ml) in either normo-osmotic (305 mosmol/l) or hyperosmotic media (405 mosmol/l). Hyperosmotic conditions were achieved by addition of NaCl (50 mmol/l), raffinose (100 mmol/l), saccharose (100 mmol/l) or urea (100 mmol/l) to the normo-osmotic Krebs–Henseleit medium. Western blot analysis was performed with antibodies specific for Cox-2 and PLA_2 as described in the Materials and methods section.

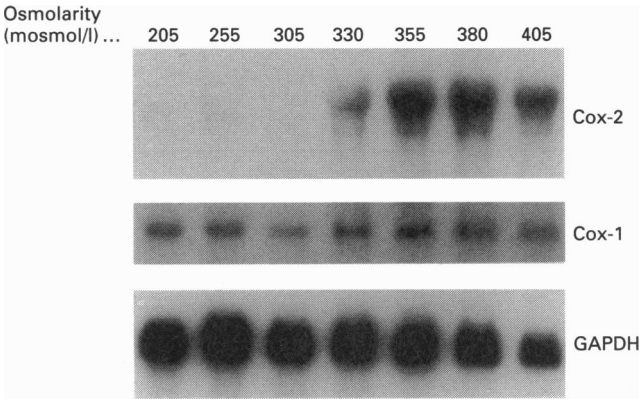

Figure 7 Osmolarity-dependent increase in mRNA levels for Cox-2, but not Cox-1, in LPS-stimulated rat Kupffer cells

Kupffer cells were incubated for 24 h with LPS (1 μg/ml) in media with different osmolarities. Osmolarity changes were performed by addition or removal of corresponding amounts of NaCl to/from the normo-osmotic Krebs–Henseleit medium (305 mosmol/l). Northern blot analysis for Cox-1, Cox-2 and GAPDH was performed as described in the Materials and methods section.

induced Cox-2 expression, however, markedly enhanced the levels of immunoreactive Cox-2 at longer times (8–24 h) of exposure. Apparently, the time-course of Cox-2 induction (Figure 4) roughly resembles that of hypertonicity-induced stimulation of PGE_2 formation (Figure 3). In the presence of LPS, induction of Cox-2 was strongly dependent upon the osmolarity of the medium (Figure 5). In the absence of LPS, only at a medium osmolarity of 405 mosmol/l, Cox-2 protein sometimes became visible (Figure 5). This effect, however, was not constant (compare Figure 5 with Figure 8). The levels of Cox-1 protein were not significantly affected by aniso-osmolarity, regardless of whether LPS was present or not (Figure 5). Similarly, the levels of immunoreactive PLA_2 were not affected by hyperosmotic exposure for 24 h (Figure 6). As shown in Figure 6, an increase in Cox-2 protein also occured when hyperosmolarity was instituted by addition of impermeant sugars such as raffinose or saccharose, but not when hyperosmolarity was induced by addition of rapidly permeating urea. In line with this, hyperosmolarity due to the addition of urea had no effect on PGE_2 production (results not shown).

The induction of Cox-2 protein in reponse to hyperosmotic/LPS exposure was accompanied by a marked increase in Cox-2, but not Cox-1, mRNA levels (Figure 7). The increase in Cox-2 mRNA occurred after about 6 h of exposure to LPS/hyperosmolarity (results not shown).

The accumulation of immunoreactive Cox-2 in response to LPS/hyperosmolarity was abolished in the presence of dexamethasone, actinomycin D or cycloheximide (Figure 8). This indicates that protein synthesis is required for the increase of immunoreactive Cox-2 in response to hyperosmolarity in LPS-treated Kupffer cells. Although indomethacin completely inhibited PGE_2 formation (Table 2), synthesis of Cox-2 protein in hypertonic media was not inhibited (Figure 8). As shown in Figure 9, hyperosmotic exposure also increased Cox-2 protein when Kupffer cells were stimulated with phorbol ester.

Cox-2 has been shown to contain four potential sites of N-glycosylation [32], and glycosylation is apparently required for the catalytic activity of Cox-2. As shown in Figure 9, endoglucosidase H treatment led to a reduction of the molecular mass of Cox-2 by approx. 5 kDa, consistent with previous reports [32]. The findings indicate that hyperosmotic exposure of LPS- or PMA-stimulated Kupffer cells leads to the induction of a highly glycosylated form of Cox-2. In line with this, addition of tunicamycin, a glycosylation inhibitor, to Kupffer cells being cultured in LPS-containing hyperosmotic medium, led to the appearence of a Cox-2 protein with a molecular mass of 65 kDa,

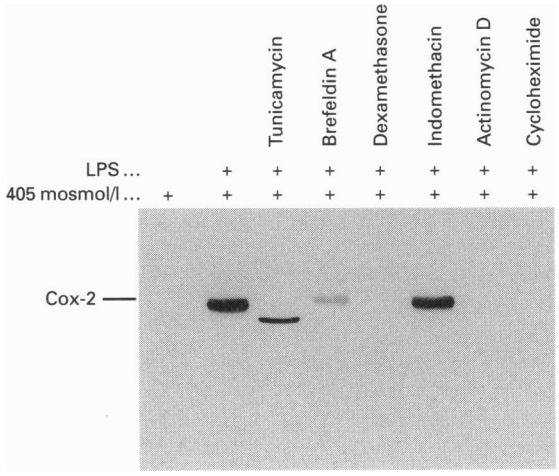

Figure 8 Effects of dexamethasone, actinomycin D, cycloheximide, tunicamycin, indomethacin and brefeldin A on Cox-2 protein expression in hypertonic LPS-stimulated rat Kupffer cells

Kupffer cells were incubated in hyperosmotic medium (405 mosmol/l) in the absence (first lane only) or presence of LPS (1 μg/ml) plus indicated inhibitors for 24 h. Western blot analysis was performed with an antibody specific for Cox-2 as described in the Materials and methods section. The following concentrations were used: tunicamycin (5 μg/ml); brefeldin A (5 μg/ml); actinomycin D (1 μg/ml); cycloheximide (10 μg/ml); indomethacin (10 μmol/l); dexamethasone (1 μmol/l).

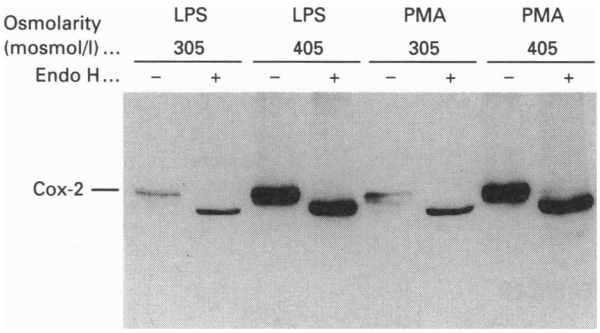

Figure 9 Effect of endoglucosidase H on Cox-2 protein expression

Kupffer cells were incubated in normo-osmotic (305 mosmol/l) or hyperosmotic (405 mosmol/l) media with LPS (1 μg/ml) or PMA (0.1 μmol/l) for 24 h. The protein samples obtained from the cells were then incubated in the presence or absence of endoglucosidase H (Endo H) for 16 h (for details see the Materials and methods section).

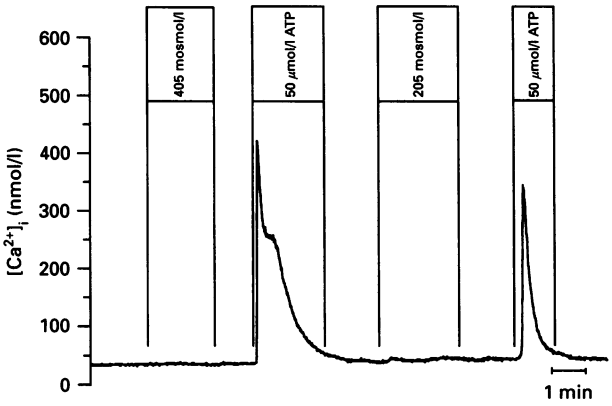

Figure 10 Effects of aniso-osmolarity and extracellular ATP on cytosolic free calcium ($[Ca^{2+}]_i$) in activated Kupffer cells measured by fura 2 fluorescence at the single-cell level

Kupffer cells were incubated in normo-osmotic (305 mosmol/l) medium with LPS (1 μg/ml) for 24 h and then loaded with fura 2 acetomethyl ester (5 μmol/l) for 30 min. Then cells were continuously superfused and subjected to single-cell fluorescence recording (for further details see the Materials and methods section). Under normo-osmotic superfusion condition, $[Ca^{2+}]_i$ was 34 ± 2 nmol/l ($n = 49$).

instead of 70 kDa, which is found in the absence of tunicamycin (Figure 8). Interestingly, treatment with tunicamycin lowered PGE_2 release by LPS/hyperosmolarity (405 mosmol/l)-stimulated Kupffer cells by about 90%. Here, PGE_2 production was 235 ± 21 pmol/24 h per 10^6 cells in the absence and 28 ± 6 pmol/24 h per/10^6 cells in the presence of tunicamycin. These data could indicate that glycosylation of Cox-2, which is induced in response to LPS/hyperosmolarity, is required for its enzymic activity. Brefeldin A, an inhibitor of protein transfer from the endoplasmic reticulum to the Golgi apparatus, did not shift the molecular mass of Cox-2 to lower values.

Effect of hyperosmotic exposure on $[Ca^{2+}]_i$

A rise in $[Ca^{2+}]_i$ is known to activate PLA_2 and to stimulate prostanoid synthesis [1]. Accordingly, we studied the effect of LPS and hyperosmotic exposure on the cytosolic free Ca^{2+} concentration in fura 2-loaded cultivated Kupffer cells at the single cell level. During normo-osmotic cultivation $[Ca^{2+}]_i$ was 34 ± 2 nmol/l ($n = 49$) in unstimulated Kupffer cells and 26 ± 4 nmol/l ($n = 12$) and 23 ± 3 nmol/l ($n = 10$) following 24 h exposure to LPS (1 μg/ml) or PMA (0.1 μmol/l) respectively. Neither in unstimulated nor in 24 h LPS- or PMA-treated cells did hyperosmotic exposure have any effect on $[Ca^{2+}]_i$, whereas extracellular ATP elicited a strong increase in $[Ca^{2+}]_i$ (Figure 10). $[Ca^{2+}]_i$ in cells exposed for 24 h to LPS/hyperosmolarity or PMA/hyperosmolarity was 36 ± 5 nmol/l ($n = 10$) or 28 ± 10 nmol/l ($n = 14$), i.e. not significantly different from the value found in control cells under hyperosmotic conditions (32 ± 7 nmol/l; $n = 14$). Thus, changes in $[Ca^{2+}]_i$ probably do not explain the hyperosmolarity-induced increase in prostanoid production by Kupffer cells.

DISCUSSION

Aniso-osmotic regulation of prostanoid formation

As shown in this paper, hyperosmotic exposure has little effect on PGE_2, D_2 and thromboxane B_2 formation in unstimulated rat Kupffer cells, but markedly enhances prostanoid formation when the cells are exposed to LPS or phorbol ester (Figure 1). Stimulation of PGE_2 formation by hyperosmotic exposure is apparently not explained by changes in the Na^+ and Cl^- activity, because it also occurs when hyperosmotic conditions are instituted by addition of impermeant sugars. In contrast to NaCl and raffinose, urea permeates plasma membranes well. Hyperosmotic conditions, induced by the addition of permeant urea to the normo-osmotic medium, had no effect on PGE_2 formation (not shown) and Cox-2 expression (Figure 6). Thus, one may speculate that it is the cell shrinkage accompanying the hyperosmotic exposure which triggers the alterations in prostanoid formation rather than the extracellular hyperosmolarity itself.

Regulation of prostanoid formation in rat liver macrophages by ambient osmolarity may add to the complex control of arachidonate metabolism. Whether the present findings are of (patho)physiological relevance remains unclear. However, a 5–10-fold increase in PGE_2 production in stimulated Kupffer cells is already seen when the extracellular osmolarity increases from its normal value of about 300 mosmol/l by just 50 mosmol/l. In clinical medicine, changes in plasma osmolarity by 100 mosmol/l above [33] or below [34] the physiological plasma osmolarity due to changes in serum Na^+ and Cl^- have been reported. Such marked aniso-osmolarities, although apparently compatible with life, induce perturbances of cerebral osmolytes, as shown by ^{1}H-magnetic resonance spectroscopy [33,34]. In fact, hypernatremia with plasma osmolarities around 350 mosmol/l is frequently found in patients with dehydration due to inadequate fluid intake or severe diarrhoea. This latter condition may be of particular relevance for the present study, because infectious diarrhoea may lead not only to hyperosmolarity but also to an enhanced endotoxin load to the liver due to a disturbance of the intestinal barrier.

Mechanism

Several mechanisms may contribute to enhanced prostanoid formation following hyperosmotic exposure; they probably involve both cyclooxygenase and PLA_2 activation. In LPS-treated Kupffer cells hyperosmolarity leads to induction of Cox-2, as shown at the level of mRNA (Figure 7) and enzyme protein (Figures 5 and 6). The increased expression of Cox-2 (Figures 4, 5, 6, 8 and 9) is sensitive to dexamethasone and inhibitors of protein and RNA synthesis (Figure 8). On the other hand,

constitutive cyclooxygenase (Cox-1) was not induced following hyperosmotic exposure (Figures 5 and 7). Several lines of evidence suggest that hyperosmolarity-induced Cox-2 induction may contribute to the increase in prostanoid formation. (i) The hyperosmolarity-induced increase in PGE_2 formation is blocked by NS-398 (Table 2), which acts as a selective Cox-2 inhibitor at the concentrations employed [24,35]. (ii) In unstimulated Kupffer cells, PGE_2 formation was only slightly stimulated by exogenous arachidonate, whereas stimulation was much more pronounced following LPS treatment (Table 3). (iii) Both the hyperosmolarity-induced increase in PGE_2 formation as well as the appearence of Cox-2 mRNA (results not shown) and immunoreactive Cox-2 required an 8 h lag period and exhibited similar time-courses (compare Figures 3 and 4). (iv) The glycosylation inhibitor tunicamycin led to the production of Cox-2 with lower molecular mass and largely inhibited the hyperosmolarity-induced increase in PGE_2 formation. Whether the glycosylation state of Cox-2 affects the affinity of the enzyme protein for association with the plasma membrane remains unclear.

Previous studies on liver parenchymal cells have identified ambient osmolarity changes as a potent modifier of gene expression [19–21]. This view can now be extended to Kupffer cells, although the signalling events underlying the hyperosmolarity-induced stimulation of Cox-2 expression are unclear. Recent studies indicate the existence of a signalling system involving the tyrosine kinase protein p38, which is activated by both LPS and hyperosmolarity [36]. This protein is related to HOG1 in yeast, however, the upstream signalling events are unknown. It remains to be established whether activation of p38 is important for the Cox-2 induction reported here.

It remains to be established whether the hyperosmolarity-induced and actinomycin D- and cycloheximide-sensitive increase of immunoreactive Cox-2 protein and the increase in Cox-2 mRNA involves an enhanced rate of gene transcription and/or mRNA stabilization. In this respect it is of interest to note that post-transcriptional events have been suggested to play a major role in the increase of Cox-2 in response to interleukin-1 [18].

Despite the above evidence for a role of Cox-2 induction in the hyperosmotic stimulation of prostanoid formation, activation of PLA_2 is likely to contribute too. Both, PLA_2 and cyclooxygenase have been described as regulatory steps and may share control in the pathway from membrane-bound arachidonate to PGE_2 [37,38]. Clearly, PLA_2 is required for hyperosmolarity-induced stimulation of PGE_2 formation, as suggested by the inhibitory effect of the PLA_2 inhibitor employed (Table 2). Furthermore, control by this enzyme is suggested by the finding that addition of exogenous arachidonate to LPS-treated Kupffer cells caused a significant increase in PGE_2 formation (Table 3). Although the levels of immunoreactive PLA_2 did not change following LPS and hyperosmotic exposure (Figure 6), short-term activity changes may well occur, because PLA_2 activity is known to be regulated by phosphorylation and Ca^{2+} [39–41] and it is not clear to what extent hyperosmolarity affects the association of the enzyme with the plasma membrane. Among the protein kinases affecting PLA_2 activity, mitogen-activated protein (MAP) kinases [39] and PKC [42] have been identified. Activation of MAP kinases following hyperosmotic exposure has been shown to occur in yeast and some mammalian cell types [43–46]; such a phenomenon could trigger activation of PLA_2 in Kupffer cells during hyperosmolarity. It is quite conceivable that the PKC inhibitor Gö 6850 also acts at the level of PLA_2 phosphorylation to inhibit PGE_2 production (Table 2). In line with this suggestion is the finding that Gö 6850 did not affect the hyperosmolarity-induced increase in Cox-2 mRNA levels (results not shown). Hyperosmotic exposure had no significant effect on the cytosolic free Ca^{2+} concentration in both LPS-treated and -untreated Kupffer cells. Thus, it is unlikely that a hyperosmolarity-induced perturbation of cellular Ca^{2+} homeostasis contributes to PLA_2 activation.

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