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Biophysical Chemistry 89 (2001) 1–12

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Coupling of inflammatory cytokine signaling pathways probed by measurements of extracellular acidification rate

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Received 3 July 2000; received in revised form 18 September 2000; accepted 18 September 2000

Abstract

There is a growing interest in the mechanisms of how cells integrate the multitude of signals that emanate during inflammatory stimuli, such as the hepatic acute phase response to burn or trauma. We have used measurements of extracellular acidification rate (ECAR) of HepG2 cells cultured on microporous membranes to probe the coupling between signaling pathways for gp130 family cytokines (interleukin-6, oncostatin M) and IL-1, each of which is considered to play a significant role in the hepatic acute phase response. We found that brief (30 min or less) exposure to any of these cytokines desensitized the HepG2 cells to subsequent exposure with the same cytokine. Furthermore, we found that this property serves as a probe of the coupling of signaling pathways: exposure to IL-1 did not desensitize the cells to exposure to OSM and vice versa. However, cells exposed to IL-6 with soluble gp80, which together share with OSM the use of gp130 as a signal transducing receptor, were subsequently unable to respond to OSM, and vice versa. Simultaneous exposure of cells to moderate concentrations (near their respective EC_{50} values) of both IL-1 and OSM resulted in synergistic effects on the ECAR, but simultaneous exposure to saturating concentrations of IL-1 and OSM resulted in a response that tracked that of OSM alone. These results suggest that the signaling pathways of IL-1 and OSM may be simultaneously activated in HepG2 cells under moderate inflammatory cytokine challenge but that the cells must prioritize their response under extreme cytokine challenges.

Keywords: Microphysiometer; Desensitization; Acute phase response; Signaling dynamics; Dose response mechanisms

Abbreviations: APP, acute phase protein; APR, acute phase response; ECAR, extracellular acidification rate; IL-1, interleukin-1; IL-6, interleukin-6; K_d, dissociation constant; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; OSM, oncostatin M; OSMRβ; receptor subunit for OSM; sgp80, soluble gp80; TNF, tumor necrosis factor

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0301-4622/01/$ - see front matter
PII: S0301-4622(00)00212-X

1. Introduction

The acute phase response (APR) is part of the inflammatory reaction to burn injury or other trauma [1–3]. A major component of the APR is a dramatic change in the protein synthetic profile of the liver hepatocytes. This change is initiated by the binding of inflammatory cytokines to their receptors expressed on hepatocytes, followed by signal transduction, activation of transcription factors, and transcriptional (and possibly post-transcriptional [4]) regulation of target genes. The acute phase response is protective and aids the body in wound healing and in combating infection, yet an overactive acute phase response may be detrimental. Markers of the acute phase response, including inflammatory cytokine levels and acute phase protein levels, have been demonstrated to correlate with adverse clinical outcomes, such as septic shock, progression to multiple organ dysfunction syndrome, and mortality [5–7]. Because a delicate balance exists between protective and deleterious functions of the acute phase response, we are interested in studying the molecular physiology of the APR with the goal of developing strategies to control it within the desired limits.

The three cytokines most closely associated with the APR are interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). The effects of the APR can be most closely replicated in cell culture by stimulating hepatocytes with IL-6, but IL-1 and TNF-α have also been shown to directly alter the expression of so-called acute phase proteins (APPs) that are up-regulated in the APR, and also to influence the effect of IL-6 on APP expression [8–12]. While these cytokines use different receptors, each one activates multiple signal transduction pathways, and some of these are known to be overlapping [13–15] (Fig. 1). It is not clear whether this overlap leads to redundancy, synergism, or antagonism.

Because signal transduction occurs rapidly and inside the cell, it is difficult to assay for signal transduction activities quantitatively and dynamically. We are interested in how hepatocytes integrate the signaling from several cytokines activating multiple signal transduction pathways to produce the acute phase response. In order to probe the coupling among cytokine signaling pathways,

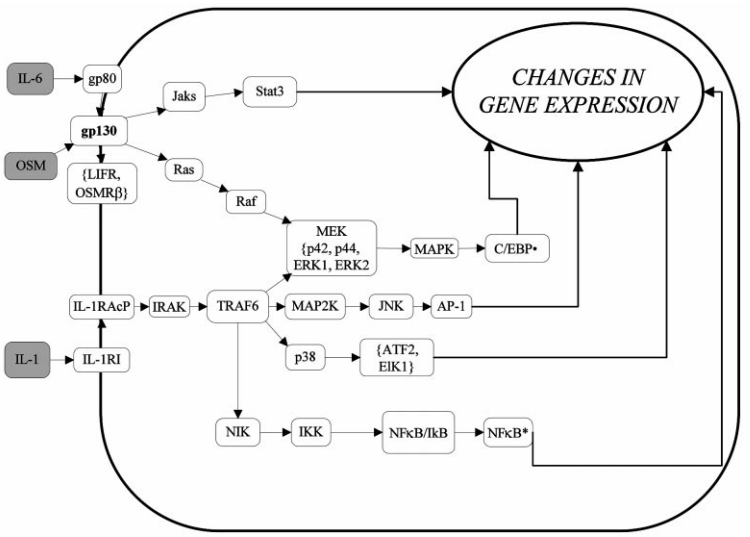

Fig. 1. Overlapping signaling pathways in the acute phase response. Changes in gene expression in response to the flood of cytokines to which hepatocytes are exposed in the acute phase response involve the interplay among several networked signaling pathways. IL-6 and OSM share the common receptor subunit gp130 and its associated signaling pathways. A common pathway (MAP kinase) exists between gp130 signaling and signaling originating from IL-1 challenge.

we exploited the fact that the activation of signal transduction pathways is energy-dependent, primarily through phosphorylation. Coupling of energy expenditure to cellular metabolism results, in many cases, in an observable increase in the rate of proton secretion, or extracellular acidification rate (ECAR), during activation [16]; this property is measured in a microphysiometer [17]. By periodically halting the perfusion of a low buffered medium past cells cultured on a microporous membrane, we can monitor the ECAR continuously, in real time, and on the time scale of minutes. We have utilized this approach to study the coupling between the signal transduction pathways activated by IL-1, IL-6 and oncostatin M (OSM), a potent member of the IL-6 family.

2. Materials and methods

2.1. Reagents and cells

All reagents were prepared using doubly-distilled, deionized water and sterile filtered. The cytokines IL-1, IL-6, leukemia inhibitory factor (LIF), OSM, and the soluble form of the IL-6 receptor, sgp80, were purchased from R&D Systems (Minneapolis, MN). Except as noted, HepG2 cells were cultured as previously described [18]. Running medium for the microphysiometer consisted of standard modified Eagle's medium (MEM) complete medium (Life Technologies, Long Island, NY) but without the sodium bicarbonate or fetal calf serum. These components were replaced with sodium chloride at 26.4 mM and charcoal filtered bovine serum albumin at 1 mg/ml (Sigma, St. Louis, MO), to maintain osmolarity and to prevent adsorption of cytokines to tubing, respectively.

2.2. Microphysiometer

ECARs were measured with a Cytosensor Microphysiometer (Molecular Devices, Sunnyvale, CA). One-milliliter aliquots of HepG2 cells were seeded in standard medium at a density of 5×10^5/ml in Cytosensor capsule cups overnight. The medium was then switched to running medium and the cups inserted into the microphysiometer system. Cells entrapped in a low volume region between two microporous membranes were perfused with low buffered medium, pre-warmed to produce a temperature of 37°C as it perfused the medium, in a continuous cycle. For 90 s, cells were perfused at a flow rate of approximately 100 μl/min, after which flow was stopped for 30 s and the cycle repeated. The decrease in pH (recorded as millivolts by the detector) was quite linear and was averaged over the stopped flow interval to obtain the ECAR. The 90 s duration per cycle of perfusion was chosen such that steady-state sensor output was re-attained in each cycle. Cells were allowed to equilibrate in the microphysiometer 1–2 h, until a steady baseline of ECAR was established (in the range of 80–120 μV/s, with a range of approx. 10 μV/s for the four capsules in a simultaneous run), before any cytokine stimulation was initiated.

The microphysiometer has two medium reservoirs for each chamber of cells, with a valve controlling the reservoir from which the cells are perfused. When a change in medium conditions (e.g. cytokine challenge) was to be initiated, the new medium was placed in the bypass reservoir for at least 10 min prior to introduction. This minimized dead volume effects and the slight variability of pH between medium in the microphysiometer and that sitting in identical vials in a sterile hood. Nonetheless, slight changes in pH were apparent as an offset in ECAR values during medium switches during some experiments. Responses after stimulation with effector or control agents were normalized to baseline values to account for the slight well-to-well variation in basal ECAR, which was generally less than 10%.

2.3. Haptoglobin secretion

Measurements of haptoglobin secretion were made essentially as described [18]. Confluent monolayers of HepG2 cells in 24-well plates were treated with cytokines for 24 h, at which time the medium was harvested from each well. All samples were assayed for the presence of the acute phase protein haptoglobin by an ELISA previ-

ously developed and described [18]. Values were normalized to that for cells receiving no cytokine.

2.4. Data analysis

For microphysiometer experiments, all values were normalized as percentage of baseline. The EC_{50} values for up-regulation were calculated based on a fit to the equation:

$$\mathrm{ECAR} = 100 + \frac{(E_{\max} - 100)c}{\mathrm{EC}_{50} + c} \quad (1)$$

where $E_{\max}$ is the maximum ECAR up-regulation and c is the concentration of cytokine. All values obtained for experiments in a 2-month period with a common batch of HepG2 cells (but over multiple passages) were used in the calculations. Thus, each data point was used in the fits, rather than a composite average for each concentration. For haptoglobin secretion experiments, the values (amounts of haptoglobin secreted in 24 h) were normalized to the secretion rate of cells receiving no cytokine and represent the mean ± S.D. from three samples of HepG2 conditioned medium, each assayed in triplicate. The analogous equation for determining the EC_{50} values for normalized haptoglobin upregulation (h) is:

$$h = 1 + \frac{(h_{\max} - 1)c}{\mathrm{EC}_{50} + c} \quad (2)$$

In both cases, the non-linear regressions were performed in Kaleida Graph (Synergy Software, Reading, PA), v. 3.0 for Windows.

3. Results

3.1. Dynamics of ECAR responses

We first investigated whether the cellular physiology of the APR could be probed using a microphysiometer. For these studies, we utilized the HepG2 cell line, which has been extensively characterized, using more traditional assays, with respect to its responsiveness to inflammatory cytokines, providing a number of insights into the molecular physiology of the acute phase response [19–21]. The HepG2 cells proved quite responsive to IL-1 and to OSM, a potent inflammatory cytokine that shares with IL-6 the use of gp130 as its signaling receptor; however, they were unresponsive to IL-6 (Fig. 2). Since IL-6 is known to stimulate changes in acute phase protein expression by HepG2 cells, this was somewhat surprising. However, a complex of IL-6 and a soluble form of its receptor, sgp80, resulted in a marked increase in ECAR. Since HepG2 cells constitutively express gp80 on their surface, the difference between IL-6 and IL-6/sgp80 exposure is presumably due to the two-step process required for IL-6 to bind gp80, followed by association with gp130 (see Section 4). OSM is able to bind gp130 directly, although other receptor subunits (LIFR and/or OSMRβ) are required for OSM to induce signal transduction [22,23].

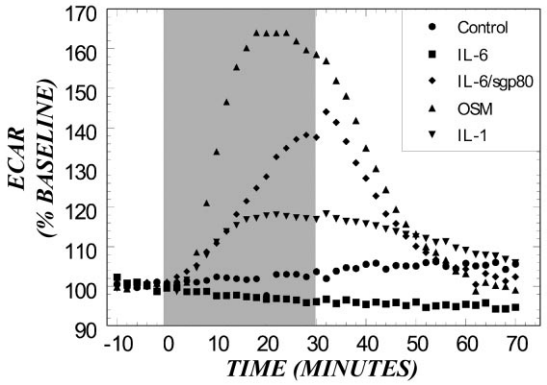

Fig. 2. ECAR is rapidly increased by HepG2 cells in response to some, but not all, inflammatory cytokines. ECAR for HepG2 cells stimulated for 30 min (shaded) with 10 ng/ml IL-1 (inverted triangles), 10 ng/ml OSM (upright triangles), 10 ng/ml IL-6 (squares), 10 ng/ml IL-6 plus 1000 ng/ml soluble gp80 (diamonds), or no cytokine (circles). All data are expressed relative to baseline values.

3.2. Desensitization and coupling of pathways

The ECAR responses reached a maximum and

began to decline over a period of 30–60 min even under continued exposure to cytokine, suggesting that the responsiveness of the cells is rapidly attenuated. Furthermore, we found that a second challenge with the same cytokine a short time after the first resulted in a greatly diminished response, i.e. the cells were desensitized with respect to the ECAR response. In order to determine whether this effect reflected a down-regulation of cytokine-specific components or of general cell signaling capability, we measured the response of HepG2 cells to a second challenge of either the original cytokine or another one to which they are responsive. We found that HepG2 cells previously exposed to 10 ng/ml IL-1 for 30 min were unable to respond to subsequent IL-1 challenge, even after 60 min without exposure (Fig. 3 and Table 1). This pattern of previous exposure did not significantly diminish the ability of the cells to respond to OSM or a combination of IL-6 and soluble gp80 (Fig. 3). These results indicate that the desensitization to IL-1 is not a loss in general ability to transduce signals or regulate acidification but is indeed particular to the IL-1 receptor and/or signal transduction pathway. The analogous result held for the converse experiment involving exposure to OSM followed by rest and a second exposure to various cytokines, in which a greatly reduced (but not abolished) response to OSM occurred with little effect on IL-1 (Table 1).

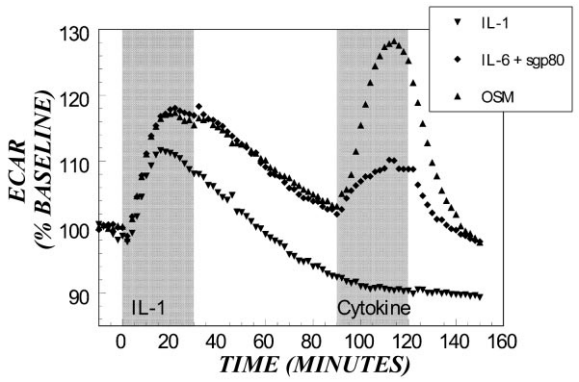

Fig. 3. Desensitization to cytokine challenge. HepG2 cells were stimulated for 30 min (shaded) with 10 ng/ml IL-1, with medium only for 60 min, then challenged with the indicated cytokines for 30 min (shaded). All concentrations for the second challenge were 10 ng/ml, except sgp80 was 1000 ng/ml. ECAR values are expressed relative to baseline before the first stimulation.

Table 1
Desensitization occurs in the ECAR response to agents acting through common signaling pathways[a]

First challenge	Second challenge	Response to second challenge
IL-1	IL-1	−
IL-1	IL-6/sgp80	+
IL-1	OSM	+
OSM	IL-1	+
OSM	IL-6/sgp80	Greatly reduced
OSM	OSM	−
IL-6/sgp80	OSM	Greatly reduced

[a] In all cases, the first challenge consisted of a 30-min incubation with the indicated cytokine. This was followed by 60 min with no cytokine and a second challenge of 30 min of the indicated cytokine. All cytokine concentrations were 10 ng/ml, except 1000 ng/ml for sgp80. The responses to the second challenge are graded in one of the following three categories: equal (±20%) to the response without prior challenge as '+', essentially abolished (less than 10% of the response without prior challenge) as '−', or significantly above zero but less than half of the response without prior challenge as 'greatly reduced'.

Desensitization of the ECAR response to cytokine stimulation appears to be specific to individual receptor activation/signal transduction pathways [24,25], suggesting that it can be used as an assay to probe the coupling of overlapping signal transduction pathways. In order to verify that the desensitization is specific to the cellular pathway and not the cytokine itself, we compared the responses of HepG2 cells exposed first to either OSM (10 ng/ml; 30 min) or a combination of IL-6 (50 ng/ml) and sgp80 (1 μg/ml). Both OSM and the IL-6/sgp80 complex utilize gp130 as a signal transducing receptor and activate Jak/Stat as well as MAP kinase signal transduction pathways [26–29] (Fig. 1). After the initial stimulation, the cells were challenged with the other cytokine, and in both cases desensitization was observed (Table 1). Thus, the desensitization was indeed at the cellular receptor/signal transduction level.

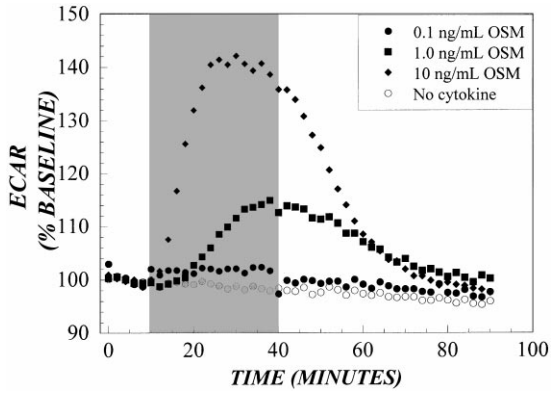

Fig. 4. Dose response of ECAR by HepG2 in response to OSM. HepG2 cells were exposed to the indicated concentrations of OSM for 30 min (shaded), and the ECAR monitored for an additional 60 min. All data are expressed relative to baseline values.

3.3. Dose response relationships

We also studied the dose response relationships of the ECAR to more quantitatively characterize the HepG2 response to OSM; frequently, the dose response of signal transduction and downstream function are related closely to that of the initial ligand binding event. The ECAR response was quite dose dependent to OSM concentrations in the range of 0.1–10 ng/ml (Fig. 4), but lower and higher concentrations resulted in no response and no additional response compared with 10 ng/ml, respectively. By varying the concentration of cytokine used to stimulate the HepG2 cells, we determined an EC_{50} for the OSM-dependent up-regulation of ECAR of 1.8 ± 0.8 ng/ml. This value compares quite well with the measured high affinity dissociation constant between OSM and HepG2 cells of 2.0 ng/ml [30]. The ECAR response to IL-1 was also dose-dependent, but with the half-maximal value occurring at much lower concentration, i.e. $EC_{50} = 0.015 \pm 0.003$ ng/ml. Because no measurable response to IL-6 was detected, we could not determine the EC_{50} for IL-6.

We also determined the EC_{50} for the functional response of these cytokines on HepG2 cells, as measured by the up-regulation of the acute phase protein, haptoglobin. OSM was found to stimulate both a greater overall up-regulation as compared to IL-6 and to do so at lower concentrations, specifically with an $EC_{50} \sim 0.6$ ng/ml for OSM and $EC_{50} \sim 4.1$ ng/ml for IL-6 (Fig. 5). The value for OSM is again close to the value for ligand dissociation, whereas the EC_{50} is much greater than the K_d for IL-6. The difference between the EC_{50} and K_d in the case of IL-6 may reflect kinetic, as opposed to thermodynamic, limitations in IL-6 binding, which could also explain the inability to detect a significant ECAR response using IL-6 alone and the different

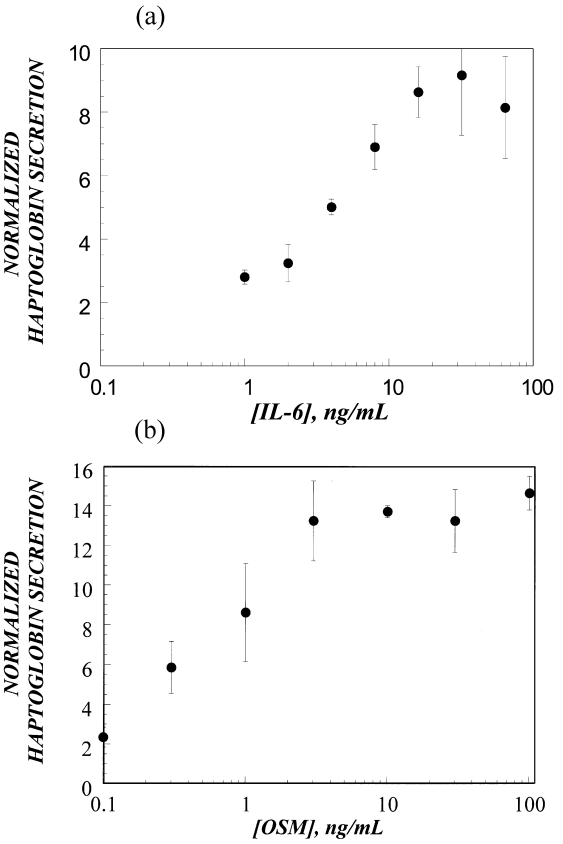

Fig. 5. Dose response of haptoglobin secretion in response to IL-6 and OSM. HepG2 cells, at 95% confluence in 24-well plates, were incubated with the indicated concentrations of (a) IL-6 or (b) OSM in a fresh change of medium for 24 h, after which their medium was harvested and assayed for haptoglobin by ELISA [18]. Values represent the mean ± S.D. ($n = 3$ wells of cells, each assayed in triplicate), normalized to cells receiving no IL-6 and no OSM.

Table 2
Dose response values for cytokine activities on HepG2 cells[a]

Assay	Value (ng/ml)		
	IL-1	IL-6	OSM
High affinity binding (K_d)	4.2	0.4	2.0
ECAR (EC_{50})	0.015 ± 0.003	ND	1.8 ± 0.8
Haptoglobin secretion (EC_{50})	ND	4.1 ± 1.0	0.61 ± 0.12

[a]Values for high affinity binding taken from the literature [30,50,51]. The EC_{50} values for microphysiometer (ECAR) measurements are based on the maximum ECAR during a 1-h period, with the indicated cytokine added during the first half-hour. The ECAR for IL-6 stimulation alone was undetectable and thus the EC_{50} was not determined (ND). Haptoglobin secretion was determined after 24-h exposure to the indicated cytokine, as described in the legend to Fig. 5. The effect of IL-1 on haptoglobin expression by HepG2 cells is small, and thus the dose response was not determined for this case.

plateau values for OSM vs. IL-6 induced up-regulation of haptoglobin. IL-1 did not exhibit a significant effect on haptoglobin secretion in these cells. Where obtainable, the K_d and EC_{50} values for ECAR response and haptoglobin up-regulation are summarized in Table 2.

3.4. Synergy of IL-1 and OSM

In the acute phase response, circulating levels of several cytokines increase, which can act simultaneously upon the hepatocytes of the liver. In order to provide a measurement of receptor activation in the presence of multiple cytokines, we challenged HepG2 cells with a combination of IL-1 and OSM. Using a combination of concentration levels near the EC_{50} values (Table 2), namely 3 ng/ml OSM and 0.03 ng/ml IL-1, a synergy in the ECAR response was observed (Fig. 6a). In fact, over the interval of the initial stimulation, the ECAR of cells receiving a combination of 3 ng/ml OSM and 0.03 ng/ml IL-1 quantitatively tracked the sum of the ECAR profiles of the cells receiving each of these cytokine levels individually. In contrast, when high concentrations (10 ng/ml each) of OSM and IL-1 were used, the effect of combined cytokines mirrored that of OSM alone (Fig. 6b).

4. Discussion

The microphysiometer is a relatively new tool that is able to provide an indication of changes in cellular metabolism resulting from receptor activation (signal transduction) on living cells on the time scale of minutes. As such, it is suited for quantitative studies of receptor activation and signal transduction dynamics that have been limited to date by the qualitative and invasive nature of signal transduction assays. Its disadvantage is that the ECAR is a property that represents an integration of a number of intracellular processes, and so its quantitative relationship to molecular and cellular parameters has yet to be conclusively elucidated [16]. In this study, we have provided some evidence that the observed ECAR of HepG2 cells in response to the inflammatory cytokines IL-1, OSM and IL-6/sgp80 is closely related to signal transduction. Furthermore, we have used a property of the ECAR response, desensitization, as a means to identify coupling between inflammatory cytokine pathways in HepG2 cells.

The ECAR of cells is generally believed to be stimulated in two ways: through the sodium-hydrogen exchanger and through an energy-dependent metabolic pathway(s) that requires glucose and is affected by activation of cellular receptors to a variety of agonists [17,31]. The former mechanism results in rapid, transient responses that peak in the first few minutes after stimulation, leading to an overshoot in the ECAR response [16,32,33]; no evidence of this behavior was observed in our experiments. For the latter mechanism, signal transduction involves the phospho-

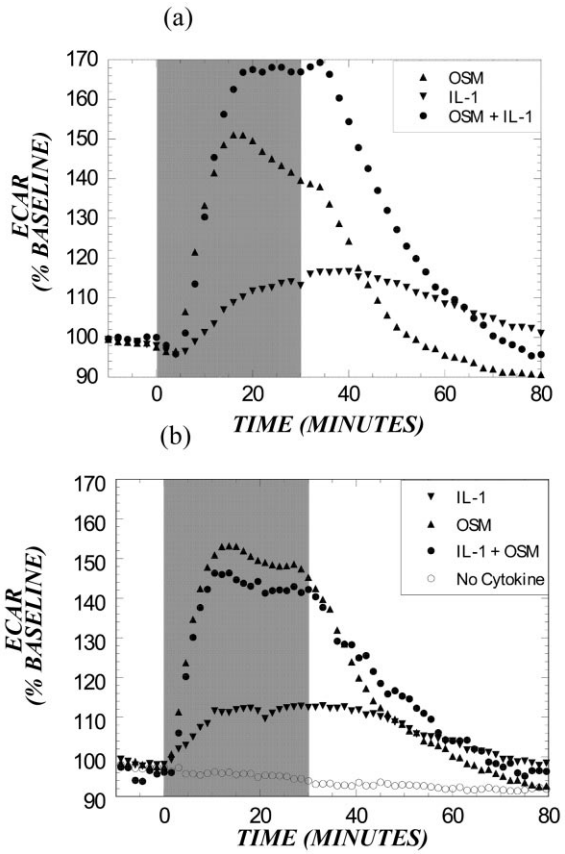

Fig. 6. Concentration dependence of synergy between IL-1 and OSM. (a) HepG2 cells were incubated for 30 min (light shading) with either 0.03 ng/ml IL-1 (inverted triangles), 3 ng/ml OSM (upright triangles), or both 0.03 ng/ml IL-1 and 3 ng/ml OSM (●). ECAR values are expressed relative to baseline before the first stimulation. (b) HepG2 cells were incubated for 30 min (light shading) with either 10 ng/ml IL-1 (inverted triangles), 10 ng/ml OSM (upright triangles), both 10 ng/ml IL-1 and 10 ng/ml OSM (●), or no cytokine (○). ECAR values are expressed relative to baseline before the first stimulation.

rylation of receptors and downstream kinases, requiring ATP, which is replenished by coupling to glycolysis and respiration. The rate limiting step is generally receptor binding, with signal transduction (phosphorylations), acid secretion, and turnover of ATP occurring much more rapidly [16]. For many cytokines and growth factors, most of the ligand binding occurs on the time scale of minutes to tens of minutes, consistent with the dynamics of the ECAR response. Likewise, assays of signal transduction in IL-1 or OSM treated cells follow similar dynamics to the observed ECAR responses [28,30]. For example, Thoma et al. [30] observed maximal MAP kinase activity in HepG2 cells at ~ 12 min of OSM stimulation for saturating concentrations of OSM; the mean time to reach a maximum ECAR in our experiments was 18 ± 3 min ($n = 9$) at 10 ng/ml. Others have also noted the correspondence of the ECAR response with ligand binding and signal transduction. For example, the maximum ECAR in the response of fibroblasts to EGF has been quantitatively correlated with receptor complex number [34]. Similarly, ECAR rates of T cells exposed to peptides have been found to correlate with the lifetimes of the MHC complexes [35]. In addition, the EC_{50} values for the response of CHO cells to human secretin correlated with the accumulation of intracellular cAMP [36].

For all of the experiments described here, the ECAR was found to transiently increase and then return to its baseline value, whether the cytokine agonist remained in the perfusion medium or not. This behavior, which represents adaptation of the extracellular acidification response to the agonist, is not universal, as other cell types and agonists have been found to lead to non-adaptive responses, i.e. the ECAR remained elevated for the duration of agonist exposure, and in some cases even after its removal [31,36]. Several mechanisms act in concert to regulate signal transduction in the IL-6/OSM and IL-1 pathways, including internalization of bound receptor complexes, translocation of downstream kinases or transcription factors that serve as substrates for phosphorylating kinases, and induction of enzymes that reverse or inhibit the actions of signaling kinases, such as the suppressors of cytokine signaling (SOCS) proteins [37,38]. We have been able to modulate the ECAR response (data not shown) with antagonists to receptors (anti-gp130 antibody and IL-1 receptor antagonist), inhibitors of dephosphorylation (sodium vanadate) and inhibitors of translocation (SN50 peptide [39]), suggesting that the ECAR response represents an interplay among all of these modules.

One possible mechanism for the modulation of

signaling activity is through the activation of multiple pathways that negatively regulate each other [40]; in fact, there is recent evidence suggesting some degree of cross-talk between the MAP kinase and Jak-STAT pathways. Recent studies have shown that inhibition of IL-6 mediated activities occurring through the STAT3 pathway in other cell types can be caused by agents that activate MAP kinase pathways [41,42]. The mechanism uncovered in these studies involved ERK2 phosphorylation of serine residues on STAT3, inhibiting its activation by tyrosine phosphorylation. For this reason, and because IL-1 and IL-6 are believed to act cooperatively in the hepatic acute phase response [1,43], we were interested in characterizing the specificity and dynamics of signaling activity in response to these cytokines. Our experiments were designed to test whether the use of shared pathways (i.e. MAP kinase) by IL-1 and OSM (Fig. 1) would inhibit the overall signal transduction initiated by these cytokines in concert. The quantitatively additive increases in ECAR by sub-maximal levels of IL-1 and OSM (Fig. 6a) suggests that this is not the case. At saturating concentrations of IL-1 and OSM, however, the response mimicked that of OSM alone. This result suggests the intriguing notion that, in the face of an overwhelming biochemical challenge, the cell must prioritize its response by rules that are set by the biochemical kinetics and thermodynamics of each pathway. However, we cannot rule out other possibilities at this time, such as a saturation of the transporters used to secrete acids from the cells.

The dose response curves for various related functions provide further clues regarding the interplay of regulatory mechanisms in the ECAR and functional response. At the receptor level, the dose response of downstream signaling can be altered by the relative kinetics of internalization versus binding. Rapid internalization leads to depletion of surface receptor complexes, which catalyze downstream signaling reactions; thus, internalization causes a shift in the dose response curves such that higher concentrations of ligand are required for a functional response, i.e $EC_{50} > K_d$. Alternatively, if internalization is relatively slow, then the functional response follows the dose response established by ligand dissociation, i.e. $EC_{50} \approx K_d$. The EC_{50} and K_d values corresponded closely for OSM, whereas in the case of IL-6, the EC_{50} for haptoglobin up-regulation was approximately an order of magnitude higher than its K_d; furthermore, no significant ECAR could be recorded for this cytokine unless its soluble receptor sgp80 was added at high concentration (Fig. 2).

Evidence from other reports supports the view that IL-6/gp80 association limits the biological effectiveness of IL-6. A number of researchers have noted enhanced activity of IL-6 in the presence of sgp80, even on cell lines that express gp80 constitutively [44,45]. For example, a fusion protein of IL-6 and its soluble receptor ('Hyper-IL-6') has been found to exhibit enhanced potency (both decreased EC_{50} and increased magnitude) in haptoglobin up-regulation as well as other IL-6 mediated responses [46]. Furthermore, the mechanism of enhancement has been found to be related to decreased internalization of Hyper-IL-6 [47]. The implication for the present work is that the kinetics of forming and maintaining a high affinity complex with signaling capability are likely limiting the effectiveness of IL-6 relative to OSM (as well as IL-6/gp80 complexes), both in terms of the EC_{50} as well as the magnitudes of the ECAR and functional responses (Figs. 1 and 4), which were greater for OSM.

Kinetics of reactions downstream of ligand-receptor binding can also influence dose response relationships. For example, cascades of enzymes in which the downstream members are acting near saturation can produce dose response curves that are shifted to lower concentrations. A well characterized pathway, both theoretically and experimentally, exhibiting this behavior is the MAP kinase pathway [48,49], which is one of the major pathways activated by IL-1 binding. Thus, it is possible that this effect is responsible for the two order of magnitude shift to a lower ECAR EC_{50} relative to the high affinity K_d for IL-1.

In summary, we have used measurements of extracellular acidification rate to probe the coupling of inflammatory cytokine pathways in HepG2 cells. The rate of activation and its decay are consistent with measurements of signaling, as

determined by tyrosine phosphorylation and direct kinase activity assays, but the microphysiometer has the advantage of providing continuous measurements in situ. In the case of OSM treatment, the dose response of the ECAR increase correlated well with that of the functional response of haptoglobin up-regulation. Many receptors are activated in complexes that form in such a way that ligand binding is not an accurate indicator of signal transduction and function; the signal transduction processes underlying the ECAR response may be more directly related to the downstream functions of interest. Furthermore, following the desensitization of cytokine-mediated signaling may be useful in the design and application of therapeutic agents designed to block the effects of cytokines. Using the microphysiometer in this way provides a novel means for assessing the coupling of responses to various cytokines. For the interaction of OSM and IL-1, the desensitization experiments showed little coupling between the receptor activation pathways. Moreover, the results suggest that cells may integrate signals from multiple cytokines quite differently depending on their concentrations. Clearly, this supposition needs to be tested by other techniques. Finally, this approach could be used for the characterization of agents to modulate the hepatic acute phase response.

Acknowledgements

The authors thank François Berthiaume and Joe Le Doux for insightful comments on earlier versions of the manuscript. This work was partially supported by a grant from the Shriners Hospitals for Children.

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