Insulin Induces Swelling-dependent Activation of the Epidermal Growth Factor Receptor in Rat Liver*

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The aim of the study was to analyze whether the proliferative effects of insulin in rat liver involve cross-signaling toward the epidermal growth factor receptor (EGFR) and whether this is mediated by insulin-induced hepatocyte swelling. Studies were performed in the perfused rat liver and in primary rat hepatocytes. Insulin (35 nmol/liter) induced phosphorylation of the EGFR at position Tyr845 and Tyr1173, but not at Tyr1045, suggesting that EGF is not involved in insulin-induced EGFR activation. Insulin-induced EGFR phosphorylation and subsequent ERK1/2 phosphorylation were sensitive to bumetanide, indicating an involvement of insulin-induced hepatocyte swelling. In line with this, hypooxosmotic (225 mosmol/liter) hepatocyte swelling also induced EGFR and ERK1/2 activation. Insulin- and hypooxosmolarity-induced EGFR activation were sensitive to inhibition by an integrin-antagonistic RGD peptide, an integrin β1 subtype-blocking antibody, and the c-Src inhibitor PP-2, indicating the involvement of the recently described integrin-dependent osmosensing/signaling pathway (Schliess, F., Reissmann, R., Reinehr, R., vom Dahl, S., and Häussinger, D. (2004) J. Biol. Chem. 279, 21294–21301). As shown by immunoprecipitation studies, insulin and hypooxosmolarity induced a rapid, RGD peptide- and integrin β1-blocking antibody and PP-2-sensitive association of c-Src with the EGFR. As for control, insulin-induced insulin receptor substrate-1 phosphorylation remained unaffected by the RGD peptide, PP-2, or inhibition of the EGFR tyrosine kinase activity by AG1478. Both insulin and hypooxosmolarity induced a significant increase in BrdU uptake in primary rat hepatocytes, which was sensitive to RGD peptide- and integrin β1-blocking antibody, PP-2, AG1478, and PD098059. It is concluded that insulin- and hypooxosmolarity-induced hepatocyte swelling triggers an integrin- and c-Src kinase-dependent EGFR activation, which may explain the proliferative effects of insulin.

Apart from its metabolic effects, insulin exerts proliferative effects in the liver and other organs (1–5). Much effort has been devoted to the understanding of insulin signaling and its complexity (6–11), which involves tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1)2 and activation of a variety of protein kinases such as mitogen-activated protein (MAP) kinases ERK1/2 and p38MAPK (3, 12). In rat liver, insulin stimulates Na+/H+ antiport and K⁺/Na⁺/2Cl⁻ co-transport, thereby inducing hepatocyte swelling (13). Evidence has been presented that insulin-induced hepatocyte swelling is an integral part of insulin signaling and mediates proteolysis inhibition by the hormone through a swelling-induced p38MAPK activation (14). Like hypooxosmotic hepatocyte swelling, insulin-induced hepatocyte swelling is sensed by the integrin system with subsequent activation of c-Src kinase and downstream MAP kinases ERK1/2 and p38MAPK (14–16). In line with this, the insulin- and hypooxosmolarity-induced inhibition of autophagic proteolysis is largely abolished in presence of an integrin-antagonistic RGD peptide or the c-Src inhibitor PP-2 (14, 15). Little is known about the contribution of insulin-induced cell swelling to the proliferative effects of the hormone (1–5). Both insulin and EGF increase DNA synthesis in hepatocytes kept under serum-free conditions (17, 18), but it is still unclear whether there is convergence of insulin- and EGF-dependent signaling.

In short term cultured hepatocytes, EGF was reported to induce IRS-1 phosphorylation in an IR-independent manner because no IR-β-subunit phosphorylation occurred (19). IRS-1-phosphorylation was followed by phosphatidylinositol 3-kinase activation (19), indicating that EGF may mimic insulin effects in hepatocytes. On the other hand, insulin (100 nmol/liter) failed to induce EGFR phosphorylation in 3-h cultured rat hepatocytes (19). However, this experimental model may not pick up swelling-dependent components of insulin signaling (20), most likely due to not yet reorganized microtubules or impaired osmosensing. The latter is achieved by hepatocellular integrin/extracellular matrix (ECM) interactions, which require either the intact three-dimensional organ structure, e.g., the intact liver, or long term hepatocyte cultures that allow for endogenous ECM synthesis. A hypooxosmolarity-induced EGFR activation was shown by immunofluorescence staining in serum-starved Swiss 3T3 fibroblast (21), but the underlying molecular mechanisms remained unclear. As shown in the present study, insulin-induced cell swelling triggers activation of the EGFR through an integrin- and c-Src kinase-dependent osmosensing/signaling pathway that triggers insulin-induced hepatocyte proliferation.

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9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.
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2 The abbreviations used are: IRS-1, insulin receptor substrate-1; ECM, extracellular matrix; EGFR, EGF receptor; IR, insulin receptor; IR-β, insulin receptor β-subunit; RGD, integrin antagonistic RGDSP hexapeptide.
**EXPERIMENTAL PROCEDURES**

**Materials**—Collagenases were from Roche Applied Science. William's E medium, collagen, insulin, and bumetanide were from Sigma-Aldrich. Penicillin and streptomycin were from Biochrom (Berlin, Germany). Fetal calf serum was from Invitrogen. The integrin antagonistic G\textsuperscript{RGD}SP peptide (RGD peptide) was from Bachem (Heidelberg, Germany). PP-2 and AG1478 were from Calbiochem.

**rabbit anti-EGFR, rabbit anti-p38MAPK, and rabbit anti-FAK** were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-phospho-ERK1/2, rabbit anti-phospho-EGFR (EGFR Tyr\textsuperscript{1045}, P) antibodies, and rabbit anti-phospho-Src family Tyr\textsuperscript{418} were from Cell Signaling (Beverly, MA). Mouse anti-phospho-c-Src-Tyr\textsuperscript{418} was from Bachem (Heidelberg, Germany). PP-2 and AG1478 were from Calbiochem. Rabbit anti-phospho-IRS-Tyr 612 was from Invitrogen. Rabbit anti-IRS-1, rabbit anti-ERK1/2, rabbit anti-Yes, and rabbit anti-Fyn were from Upstate Biotechnology (Lake Placid, NY). Integrin-blocking antibodies anti-integrin \textsuperscript{\textbeta} 1 (clone 6S6) (22), anti-integrin \textsuperscript{\textbeta} 3 (clone B3A) (23), and anti-integrin oV\textbeta} 5 (clone P1F6) (24) were from Millipore.

Protein A/G-agarose was obtained from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated anti-mouse IgG and protein assay were from Bio-Rad. The enhanced chemiluminescence detection kit was from Amersham Bio-.

**FIGURE 1.** Insulin-induced EGFR phosphorylation in perfused rat liver. Rat livers were perfused as described under “Experimental Procedures.” When indicated, RGD peptide (10 \textmu mol/liter), PP-2 (250 nmol/liter), or AG1478 (1 \textmu mol/liter) was added 30 min prior to the insulin institution (35 nmol/liter) to the perfusate to inhibit integrins, c-Src or EGFR tyrosine kinase activity, respectively. Liver samples were taken at the time points indicated, and phosphorylation of EGFR Tyr\textsuperscript{845}, Tyr\textsuperscript{1045}, and Tyr\textsuperscript{1173} was analyzed by use of phospho-specific antibodies. Total EGFR served as loading control. Representative Western blots of three independent perfusion experiments are shown. Within 5 min insulin induced an RGD peptide- and PP-2-sensitive phosphorylation of EGFR residues Tyr\textsuperscript{845} and Tyr\textsuperscript{1173}, whereas no phosphorylation at position Tyr\textsuperscript{1045} is detectable within 60 min. AG1478 blunted insulin-induced Tyr\textsuperscript{1173} phosphorylation, whereas Tyr\textsuperscript{845} phosphorylation remained unchanged, suggestive of an EGFR Tyr\textsuperscript{845} transphosphorylation leading to EGFR Tyr\textsuperscript{1173} autophosphorylation.

**FIGURE 2.** Insulin-induced MAP kinase activation in perfused rat liver. Rat livers were perfused as described under “Experimental Procedures.” When indicated, RGD peptide (10 \textmu mol/liter), PP-2 (250 nmol/liter), or AG1478 (1 \textmu mol/liter) was added 30 min prior to insulin (35 nmol/liter) to the perfusate. Liver samples were taken at the time points indicated and phosphorylation of ERK-1/2 (A) and p38MAPK (B) was analyzed by use of phospho-specific antibodies. Total ERK-1/2 or p38MAPK, respectively, served as loading controls. Representative Western blots were analyzed densitometrically. Phosphorylation level at t = 0 min (i.e. immediately before insulin addition) was arbitrarily set to 1. Representative blots and statistics of at least three independent perfusion experiments are shown. A, insulin induced a significant increase in ERK phosphorylation at t = 5 and t = 15 min (#, p < 0.05), which was significantly inhibited by RGD peptide, PP-2, and AG1478 (*, p < 0.05). B, insulin induced a significant increase (#, p < 0.05) in p38MAPK phosphorylation at t = 5 min, which was significantly inhibited by RGD peptide and PP-2 (*, p < 0.05), but not by AG1478 (p > 0.05; n.s., not significant). Error bars, S.E. of the mean.
All other chemicals were from Merck at the highest quality available.

**Primary Hepatocyte Preparation and Culture**—As described previously (25), hepatocytes were isolated from livers of male Wistar rats fed ad libitum with a standard diet by a collagenase perfusion technique. Aliquots of $1.5 \times 10^6$ hepatocytes were plated on collagen-coated 6-well culture plates (Falcon) and cultured as published recently (25) for 48 h, unless indicated otherwise, before the respective experiments were started. Osmolarity changes were performed by the appropriate addition or removal of NaCl from the medium. The viability of the hepatocytes was >95% as assessed by trypan blue exclusion.

**Liver Perfusion**—The experiments were approved by the responsible local authorities. Livers from male Wistar rats (120–150 g body mass), fed a standard chow, were perfused as described previously (26) in a nonrecirculating manner.

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**Insulin and EGFR Activation**

![Insulin-induced hepatocyte swelling triggers EGFR and MAP kinase activation.](image-url)

**A**

|       | Insulin | + Bumetanide |
|-------|---------|--------------|
| 0 min | ![Image] | ![Image] |
| 5 min | ![Image] | ![Image] |
| 15 min| ![Image] | ![Image] |
| 30 min| ![Image] | ![Image] |
| 60 min| ![Image] | ![Image] |

**B**

|       | Insulin | + Bumetanide |
|-------|---------|--------------|
| 0 min | ![Image] | ![Image] |
| 5 min | ![Image] | ![Image] |
| 15 min| ![Image] | ![Image] |
| 30 min| ![Image] | ![Image] |
| 60 min| ![Image] | ![Image] |

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**FIGURE 3.** Insulin-induced hepatocyte swelling triggers EGFR and MAP kinase activation. Rat livers were perfused as described under “Experimental Procedures.” When indicated, bumetanide (5 μmol/liter) was added 30 min prior to insulin addition (35 nmol/liter) to the perfusate to inhibit insulin-induced hepatocyte swelling by blocking the Na+/K+/2Cl− co-transporter. Liver samples were taken at the time points indicated, and phosphorylation of EGFR Tyr845, Tyr1015, and Tyr1173 (A) and phosphorylation of the MAP kinases ERK1/2 and p38MAPK (B) was analyzed using phospho-specific antibodies. Total EGFR, ERK1/2, and p38MAPK served as respective loading control. Western blots were analyzed densitometrically. Phosphorylation level at t = 0 min (i.e. immediately before insulin addition) was arbitrarily set to 1. Representative Western blots of three independent perfusion experiments are shown. Bumetanide blunted insulin-induced EGFR Tyr845, Tyr1015, ERK1/2, and p38MAPK phosphorylation, indicating the insulin-induced hepatocyte swelling is a prerequisite for activation of those kinases. Error bars, S.E. of the mean.
perfusion medium was the bicarbonate-buffered Krebs-Henseleit saline plus 1-lactate (2.1 mm) and pyruvate (0.3 mm) gassed with $O_2/CO_2$ (95/5 v/v). The temperature was 37 °C. In normoosmotic perfusions, the osmolarity was 305 mosmol/liter. Hypoosmotic exposure (225 mosmol/liter) was performed by lowering the NaCl concentration in the perfusion medium. The osmolarity was 305 mosmol/liter. For hypoosmotic perfusions, the osmolarity was 305 mosmol/liter.

**Insulin and EGFR Activation**

Rat livers were perfused as described under "Experimental Procedures." When indicated, RGD peptide (10 μmol/liter), PP-2 (250 nmol/liter), AG1478 (1 μmol/liter) (A) or bumetanide (5 μmol/liter) (B) were added 30 min prior to the institution of hypoosmolarity (225 mosmol/liter). Liver samples were taken at the time points indicated, and phosphorylation was routinely monitored with a pressure transducer (Hugo Sachs Electronics, Hugstetten, Germany) (14–16). If not stated otherwise, the compounds used in this study did not affect portal perfusion pressure.

**Western Blot Analysis**—At the end of the incubations, the medium was removed, and the cells were washed briefly with phosphate-buffered saline (PBS) and immediately lysed. Samples were transferred to SDS/PAGE, and proteins were then blotted to nitrocellulose membranes using a semidry transfer apparatus (GE Healthcare) as recently described (25, 27). Blots were blocked for 2 h in 5% (w/v) BSA-containing 20 mmol/liter Tris, pH 7.5, 150 mmol/liter NaCl, and 0.1% Tween 20 (TBS-T) and then incubated at 4 °C overnight with the respective first antibody (antibodies used: anti-phospho-EGFR Tyr845, Tyr1045, Tyr1173 and anti-phospho-c-Src-Tyr418 (1:2,500); anti-c-Src, anti-phospho-IRS-1-Tyr612, anti-IRS-1, anti-phospho-Src family-Tyr418, and anti-phospho-FAK (1:5,000); anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38MAPK, anti-p38MAPK, anti-EGFR, anti-FAK, anti-Yes, and anti-Fyn (1:10,000)). Following washing with TBS-T and incubation with horseradish peroxidase-coupled anti-mouse, anti-sheep, or anti-rabbit IgG antibody (all diluted 1:10,000) at room temperature for 2 h, respectively, the blots were washed extensively and developed using enhanced chemiluminescent detection (Amersham Biosciences). Blots were exposed to Kodak X-OMAT AR-5 film (Eastman Kodak Co., Rochester, NY).

**Immunoprecipitation**—Hepatocytes were harvested in lysis buffer as recently published (27). Equal protein amounts (200 μg) of each sample were incubated for 2 h at 4 °C with polyclonal rabbit anti-EGFR, anti-Yes, or anti-Fyn antibodies (dilution 1:100; Santa Cruz Biotechnology) to immunoprecipitate EGFR, Yes, or Fyn, respectively.
Insulin and EGFR Activation

Detection of Primary Rat Hepatocyte Proliferation—Hepatocyte proliferation was measured using a colorimetric BrdU cell proliferation assay (Roche Applied Science). Therefore, primary rat hepatocytes were cultured on collagen-coated flat-bottomed 96-well microtiter plates, respectively, for up to 48 h. The culture medium was removed and replaced by culture medium containing BrdU. For the respective samples, BrdU incorporation was determined according to the manufacturer’s recommendations.

Statistics—Results from at least three independent experiments are expressed as means ± S.E. of the mean. n refers to the number of independent experiments. Results were analyzed using Student’s t test: p < 0.05 was considered statistically significant.

RESULTS

Insulin-induced EGFR Activation in Perfused Rat Liver—Insulin (35 nmol/liter) induced within 5 min phosphorylation of the EGFR tyrosine residue Tyr1045 (Fig. 1), a known Src-inducible EGFR trans-activation site (30), which is known to induce EGFR tyrosine kinase activity (31). In line with this also, phosphorylation of the EGFR residue Tyr1173, a known EGFR autophosphorylation target (32), became detectable within 5 min (Fig. 1). No phosphorylation of EGFR residue Tyr1045, which represents an EGFR internalization site (33, 34), was observed, suggestive of ligand (EGF)-independent EGFR activation. As for control, EGF was shown to induce EGFR Tyr1045 phosphorylation (supplemental Fig. 1). AG1478, an inhibitor of EGFR tyrosine kinase activity, largely abolished insulin-induced EGFR autophosphorylation at Tyr1173, but not phosphorylation at Tyr845, indicating that insulin induces an EGFR trans-activation by another kinase.

Insulin (35 nmol/liter) induced within 5 min an activation of ERKs (Fig. 2A) and p38MAPK (Fig. 2B), which was recently shown to be mediated by insulin-induced hepatocyte swelling (15, 35). In line with this, inhibition of the integrin system using the integrin antagonistic RGD peptide or inhibition of c-Src kinase activity by PP-2 inhibited both insulin-induced ERK and p38MAPK activation (Fig. 2). Because EGFR has been reported to activate ERKs (for reviews, see 36, 37), it was tested whether inhibition of EGFR tyrosine kinase activity might influence insulin-induced ERK activation. AG1478 significantly inhibited insulin-induced ERK activation (Fig. 2A, p < 0.05), but not insulin-induced p38MAPK activation (Fig. 2B, p > 0.05; n.s. = not significant). Error bars, S.E. of the mean.

FIGURE 5. Hypoosmolarity-induced MAP kinase activation in perfused rat liver. Rat livers were perfused as described under “Experimental Procedures.” When indicated, RGD peptide (10 μmol/liter), PP-2 (250 nmol/liter), or AG1478 (1 μmol/liter) was added 30 min prior to the institution of hypoosmolarity (225 mosmol/liter). Liver samples were taken at the time points indicated, and phosphorylation of ERK1/2 (A) and p38MAPK (B) was analyzed by use of phospho-specific antibodies. Total ERK1/2 or p38MAPK, respectively, served as loading control. Western blots were then analyzed densitometrically. Phosphorylation level at t = 0 min was arbitrarily set as 1. Representative blots and statistics of three independent perfusion experiments are shown. A, hypoosmolarity induced a significant increase in ERK phosphorylation at t = 5, 15 and 30 min (#, p < 0.05), which was significantly inhibited by RGD peptide, PP-2, and AG1478 (*, p < 0.05). B, hypoosmolarity induced a significant increase in p38MAPK phosphorylation at t = 5 and t = 15 min (#, p < 0.05), which was significantly inhibited by RGD peptide and PP-2 (*, p < 0.05), but not by AG1478 (p > 0.05; n.s. = not significant). Error bars, S.E. of the mean.
K\(^+\)/2Cl\(^-\) co-transporter (38) and of insulin-induced hepatocyte swelling (13, 35), were performed. As shown in Fig. 3, both insulin-induced EGFR phosphorylation (Fig. 3A) as well as ERK and p38MAPK activation were largely abolished in presence of bumetanide (Fig. 3B, p < 0.05). These data suggest that not only insulin-dependent MAP kinase activation, but also EGFR phosphorylation depend on insulin-induced hepatocyte swelling.

This is further supported by the findings that inhibition of the integrin system using an RGD motif-containing hexapeptide (10 \(\mu\)mol/liter) or inhibition of c-Src by PP-2 (250 nmol/liter) abolished the otherwise observed insulin-induced EGFR phosphorylation at positions Tyr\(^{845}\) and Tyr\(^{1173}\), respectively (Fig. 1). The data strongly suggest that insulin mediates via bumetanide-sensitive cell swelling (35), swelling-dependent integrin and subsequent c-Src kinase activation (15) followed by trans-activation of the EGFR by phosphorylation at Tyr\(^{845}\), which is a known target of c-Src (30, 31).

**Hypotonic EGFR Activation in Perfused Rat Liver**—In line with a swelling-induced EGFR activation by insulin, hypotonic cell swelling also induced within 5 min an activation of the EGFR in the perfused rat liver (Fig. 4). The EGFR phosphorylation profile was similar to that observed in response to insulin (i.e. phosphorylation at Tyr\(^{845}\) and Tyr\(^{1173}\), but not at Tyr\(^{1045}\); compare Figs. 1 and 4). Hypoosmolarity-induced EGFR phosphorylation was sensitive to inhibition of the integrin system by an RGD peptide and PP-2, whereas AG1478 inhibited only EGFR autophosphorylation at Tyr\(^{1173}\), but not phosphorylation at Tyr\(^{845}\) (Fig. 4). As for control and in contrast to insulin-induced EGFR activation (Fig. 3A), bumetanide did not affect hypoosmotically induced EGFR phosphorylation (Fig. 4B).

In line with previous data (15, 16) and similar to the findings with insulin, hypoosmolarity led to an RGD peptide- and PP-2-sensitive activation of ERKs and p38MAPK (Fig. 5), whereas inhibition of EGFR tyrosine kinase activity by AG1478 only abolished hypoosmotic EGFR ERK but not p38MAPK activation (Fig. 5). These findings suggest an involvement of EGFR in hypoosmotic ERK, but not p38MAPK activation.

**Hepatocyte Swelling Triggers EGFR/c-Src Kinase Association**—Swelling-induced EGFR trans-activation may require an EGFR/c-Src association because hepatocyte swelling by either insulin or hypoosmolarity induces an integrin- and FAK-mediated activation of c-Src (15, 16), but not of Yes or Fyn (supplemental Fig. 2). EGFR was immunoprecipitated from insulin-treated (Fig. 6, A and C) or hypoosmotically perfused rat liver samples (Fig. 6B). As shown in Fig. 6, both exposure to either insulin (Fig. 6, A and C) or hypoosmolarity (Fig. 6B) induced within 5 min an EGFR/c-Src association. In contrast, no association of the EGFR with either Fyn or Yes, i.e. other members of the Src kinase family, was observed (supplemental Fig. 3). Whereas inhibition of the EGFR tyrosine kinase activity by AG1478 did not affect insulin- (Fig. 6A) or hypoosmolarity-induced EGFR/c-Src association (Fig. 6B), prevention of insulin-induced hepatocyte swelling by bumetanide, inhibition of integrin-dependent cell volume sensing by an RGD peptide, or inhibition of downstream c-Src kinase by PP-2 largely prevented the otherwise observed EGFR/c-Src association (Fig. 6). These data suggest that insulin-induced EGFR trans-activation requires hepatocyte swelling, integrin-mediated c-Src activation, and subsequent EGFR/c-Src association.

As for control, insulin-induced phosphorylation of IRS-1 was not affected by the RGD peptide, PP-2, AG1478 (supplemental Fig. 4) or bumetanide (35). This is in line with the previous demonstration that RGD peptide and PP-2 were also without effect on Tyr\(^{1188}\) phosphorylation of the \(\beta\)-subunit of the insulin receptor (IR-\(\beta\)) (15) and that hypoosmotic hepatocyte swelling did not induce IR-\(\beta\) or IRS-1 tyrosine phosphorylation (35).

**Insulin-induced Proliferation in Primary Rat Hepatocytes**—EGFR/c-Src association and EGFR activation in response to insulin (100 nmol/liter) or hypoosmolarity (205 mosmol/liter) were also found in primary rat hepatocytes, which were kept in culture for 48 h (Fig. 7, A and B).

To determine which RGD peptide-sensitive integrin subtype is responsible for swelling-induced EGFR activation, subtype-specific integrin-blocking antibodies, i.e. anti-integrin \(\beta 1\) (clone 656) (22), anti-integrin \(\beta 3\) (clone B3A) (23), and anti-integrin \(\alpha V\beta 5\) (clone P1F6) (24), were used (each 1 \(\mu\)g/ml). As shown in Fig. 7C, anti-integrin \(\beta 3\) antibodies inhibited insulin- and hypoosmotic-induced EGFR activation, whereas both anti-integrin \(\beta 3\) and anti-integrin \(\alpha V\beta 5\) antibodies were ineffective. As for control, EGF-induced EGFR activation was not affected by the integrin-blocking antibodies used.

![FIGURE 6. Insulin- and hypoosmolarity-induced EGFR/c-Src association in perfused rat liver](image-url)
Insulin and EGFR Activation

A

Insulin

|            | 0 min | 5 min | 15 min | 30 min | 60 min |
|------------|-------|-------|--------|--------|--------|
| P-EGFR-Y945|       |       |        |        |        |
| P-EGFR-Y1045|     |      |        |        |        |
| P-EGFR-Y1173|     |      |        |        |        |
| total EGFR |       |       |        |        |        |

205 mosmol/L

|            | 0 min | 5 min | 15 min | 30 min | 60 min |
|------------|-------|-------|--------|--------|--------|
| P-EGFR-Y945|       |       |        |        |        |
| P-EGFR-Y1045|     |      |        |        |        |
| P-EGFR-Y1173|     |      |        |        |        |
| total EGFR |       |       |        |        |        |

B

IP: EGFR

WB: Src

WB: EGFR

C

|          | control | β1 - ab | β3 - ab | αVβ5 - ab |
|----------|---------|---------|---------|-----------|
| EGFR     | control | 305 mosmol/L | EGF | control | 305 mosmol/L | EGF | control | 305 mosmol/L | EGF | control | 305 mosmol/L | EGF |
| P-EGFR-Y945|       |         |        |          |
| P-EGFR-Y1045|     |       |        |          |
| P-EGFR-Y1173|     |       |        |          |
| total EGFR |       |         |        |          |

IP: EGFR

WB: Src

WB: EGFR

D

EGF

Insulin

205 mosmol/L

405 mosmol/L

0 min

60 min

120 min

E

relative BrdU-incorporation

|          | control | Insulin | 300 mosmol/L | EGF | control | 300 mosmol/L | EGF | control | 300 mosmol/L | EGF | control | 300 mosmol/L | EGF |
|----------|---------|---------|--------------|-----|---------|--------------|-----|---------|--------------|-----|---------|--------------|-----|---------|--------------|-----|
|          |         |         |              |     |         |              |     |         |              |     |         |              |     |         |              |     |
|          |          | *       | *             |     | *       | *             |     | *       | *             |     | *       | *             |     | *       | *             |     |
|          | n.s.    | n.s.    | n.s.          |     | n.s.    | n.s.          |     | n.s.    | n.s.          |     | n.s.    | n.s.          |     | n.s.    | n.s.          |     |

* p < 0.05
# p < 0.01
n.s. non-significant
To determine whether the EGFR activated by either insulin (100 nmol/liter) or hypoosmolarity (205 mosmol/liter) is a subject for internalization as has been reported in EGF-treated cells (34, 39), EGFR was stained immunocytochemically in primary rat hepatocytes. As shown in Fig. 7D, both insulin and hypoosmolarity induced EGFR internalization similar to that induced by EGF (10 ng/ml), whereas hyperosmolarity (405 mosmol/liter) induced EGFR enrichment at the plasma membrane as described previously (39).

To analyze insulin- and hypoosmolarity-induced hepatocyte proliferation, BrdU uptake measurements were performed. As shown in Fig. 7E, hypoosmolarity (205 mosmol/liter) as well as insulin (100 nmol/liter) increased BrdU uptake significantly within 48 h, suggestive of a stimulation of hepatocyte proliferation. This swelling-induced hepatocyte proliferation by either insulin (100 nmol/liter) or hypoosmolarity (205 mosmol/liter) was sensitive to inhibition of integrin β1 subtype (RGD peptide, 100 μmol/liter; anti-integrin β1 antibody, 1 μg/ml), c-Src (PP-2, 10 μmol/liter), EGFR tyrosine kinase activity (AG1478, 5 μmol/liter) and ERK1/2 (PD98059, 10 μmol/liter), indicating that swelling-induced EGFR activation mediates hepatocyte proliferation and involves integrin β1, c-Src, EGFR, and ERK. In addition, insulin-induced hepatocyte proliferation was shown to be inhibited by bumetanide (10 μmol/liter), indicating that insulin-induced hepatocyte swelling is necessary for insulin-induced hepatocyte proliferation.

**DISCUSSION**

Proliferative effects of insulin in liver and other organs were repeatedly described in the past (1–5, 17, 18), but it remained unclear whether EGFR-mediated signaling is involved in these processes. One single study on 3-h cultured primary rat hepatocytes revealed no EGFR activation within 30 s of insulin stimulation (100 nmol/liter) (19), whereas EGF was shown to induce phosphorylation of IRS-1 but not of the IR-β-subunit (19).

As shown in the present study, within 5 min insulin induces an activating EGFR tyrosine phosphorylation at position Tyr^845 and an AG1478-sensitive EGFR Tyr^1173 autophosphorylation.

**FIGURE 7.** Insulin- and hypoosmolarity-induced EGFR activation and BrdU incorporation in primary rat hepatocytes. Primary rat hepatocytes were isolated as described under “Experimental Procedures” and seeded on collagen-coated wells for 48 h. Insulin- and hypoosmolarity-induced EGFR phosphorylation (A–C) and internalization (D) in primary hepatocytes are shown. Cells were incubated with insulin (100 nmol/liter), hypoosmolarity (205 mosmol/liter), or EGF (50 ng/ml) for the indicated time periods and then analyzed for EGFR phosphorylation at Tyr^845, Tyr^1015, and Tyr^1173 by use of phospho-specific antibodies. Total EGFR served as loading control. Representative Western blots of three independent experiments are shown. Insulin (A) and hypoosmolarity (B) induced EGFR phosphorylation at Tyr^845 and Tyr^1173, but not at Tyr^1015 and EGFR/c-Src association. C, insulin-, hypoosmolarity- and EGF-induced EGFR phosphorylation and EGFR/c-Src association were tested for integrin blocking antibody sensitivity. Anti-integrin β1 subtype antibody (β1-ab, 1 μg/ml) inhibited insulin-induced (100 nmol/liter, 5 min) and hypoosmolarity-induced (205 mosmol/liter, 5 min) EGFR phosphorylation and EGFR/c-Src association, whereas anti-β3 (β3-ab, 1 μg/ml) and anti-αvβ5 subtype antibodies (αvβ5-ab, 1 μg/ml) were ineffective. As for control, EGF-induced (50 ng/ml, 5 min) EGFR phosphorylation was not affected by the used integrin-blocking antibodies. In another set of experiments, hepatocytes were cultured on glass coverslips and immunostained for EGFR expression as described under “Experimental Procedures.” EGFR localization was visualized by confocal laser scanning microscopy. D, insulin (100 nmol/liter) and hypoosmolarity (205 mosmol/liter) induced an EGFR internalization comparable with that induced by EGF (34, 39), whereas hyperosmolarity (405 mosmol/liter) induced EGFR enrichment at the plasma membrane (39). E, insulin- and hypoosmolarity-induced proliferation of primary hepatocytes is shown. After 48 h of cell culture, culture medium was removed and replaced by culture medium containing BrdU. Then, hepatocytes were stimulated for 48 h with normosmotic control medium (305 mosmol/liter), insulin (100 nmol/liter), hypoosmolarity (205 mosmol/liter), or EGF (50 ng/ml) and then analyzed for BrdU uptake. When indicated, bumetanide (10 μmol/liter), RGD peptide (100 μmol/liter), PP-2 (10 μmol/liter), AG1478 (5 μmol/liter), or PD98059 (10 μmol/liter) was instituted 30 min prior to insulin, hypoosmolarity, or EGF incubation. In addition, integrin blocking antibodies, i.e. anti-β1, anti-β3, and anti-αvβ5 antibodies (each 1 μg/ml), respectively, were used to inhibit the respective integrin subtypes. BrdU uptake in hepatocytes kept in normosmotic control medium was arbitrarily set to 1. Statistical analyses of at least five independent experiments for each condition are shown. No significant inhibition compared with insulin, hypoosmolarity, or EGF incubation is indicated by n.s. (p > 0.05). Insulin, hypoosmolarity, and EGF induced a significant increase in hepatocyte proliferation by means of BrdU uptake (A, p < 0.05). Insulin- and hypoosmolarity-induced hepatocyte proliferation was significantly inhibited (*, p < 0.05) by RGD, anti-integrin β1, PP-2, AG1478, and PD98059. Because the insulin-induced proliferation was also bumetanide-sensitive, these data suggest a swelling-dependent, integrin β1, s-Src-, EGF- and ERK-mediated hepatocyte proliferation upon either insulin or hypoosmotic stimulation. Error bars, S.E. of the mean.
swelling-induced EGFR activation are due to different co-signals such as oxidative stress and JNK activation, which occur in response to hepatocyte shrinkage but not hepatocyte swelling (27, 28; for review, see 12, 37). However, the possibility is not excluded that the cell volume-dependent, differential engagement of c-Src and Yes in EGFR activation will contribute.

In line with the literature, both insulin and hypoosmolarity induce the activation of ERKs and p38MAPK, which is sensitive to inhibition of the integrin system and c-Src (Figs. 2 and 5) (15, 16). Interestingly, inhibition of EGFR tyrosine kinase activity by AG1478 largely abolished the insulin- or hypoosmolarity-induced ERK activation but had no effect on p38MAPK activation. These findings suggest an involvement of the EGFR tyrosine kinase activity in swelling-induced ERK but not p38MAPK activation.

ERKs are known downstream targets of the EGFR and mediators of proliferative signaling (for review, see 36, 37). In line with this, insulin and hypoosmolarity induced an AG1478and PD098059-sensitive hepatocyte proliferation, suggestive of an involvement of the EGFR and ERKs. Swelling-mediated effects of insulin on liver function require an intact cytoskeleton (20) and the physiological interaction between the integrin system and extracellular matrix (ECM) proteins (11, 12). These prerequisites and the fact that insulin-induced net K+ uptake takes several minutes (for review, see 12) may explain why an insulin-induced activation of the EGFR was not found in 3-h cultured hepatocytes within 30 s of insulin exposure (19), whereas the present study gives unequivocal evidence of an insulin-induced and swelling-dependent EGFR activation.

Hypoosmotic EGFR activation was also suggested to occur in serum-starved Swiss 3T3 fibroblasts (21). As shown in the present study, swelling-induced EGFR activation requires integrin- and c-Src signaling and can increase via ERKs hepatocyte proliferation. It is, therefore, an interesting speculation whether an altered ECM composition, as it is found in the fibrotic or cirrhotic liver, may confer resistance against insulin-induced and EGFR-mediated proliferation thereby affecting liver regeneration. The present data underline the importance of hepatocyte swelling as a mediator of insulin actions. This may explain why hyperosmotic conditions, cell dehydration, loop diuretics, and possibly altered ECM composition can trigger insulin resistance.

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