

Human Keratinocytes Respond to Extracellular UTP by Induction of Hyaluronan Synthase 2 Expression and Increased Hyaluronan Synthesis*

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The release of nucleotides into extracellular space is triggered by insults like wounding and ultraviolet radiation, resulting in stimulatory or inhibitory signals via plasma membrane nucleotide receptors. As similar insults are known to activate hyaluronan synthesis we explored the possibility that extracellular UTP or its breakdown products UDP and UMP act as mediators for hyaluronan synthase (*HAS*) activation in human epidermal keratinocytes. UTP increased hyaluronan both in the pericellular matrix and in the culture medium of HaCaT cells. 10–100 μM UTP strongly up-regulated *HAS2* expression, although the other hyaluronan synthases (*HAS1*, *HAS3*) and hyaluronidases (*HYAL1*, *HYAL2*) were not affected. The *HAS2* response was rapid and transient, with the maximum stimulation at 1.5 h. UDP exerted a similar effect, but higher concentrations were required for the response, and UMP showed no stimulation at all. Specific siRNAs against the UTP receptor P2Y₂, and inhibitors of UDP receptors P2Y₆ and P2Y₁₄, indicated that the response to UTP was mediated mainly through P2Y₂ and to a lesser extent via UDP receptors. UTP increased the phosphorylation of p38, ERK, CREB, and Ser-727 of STAT3 and induced nuclear translocation of pCaMKII. Inhibitors of PKC, p38, ERK, CaMKII, STAT3, and CREB partially blocked the activation of *HAS2* expression, confirming the involvement of these pathways in the UTP-induced *HAS2* response. The present data reveal a selective up-regulation of *HAS2* expression by extracellular UTP, which is likely to contribute to the previously reported rapid activation of hyaluronan metabolism in response to tissue trauma or ultraviolet radiation.

Cells subjected to mechanical pressure, osmotic shock, bacterial infection, chemical irritation, UV radiation, and tissue wounding rapidly release nucleotides like UTP, UDP, ATP, and ADP to the extracellular space. The nucleotide release acts as an alarm signal initiating cellular defense mechanisms (1–9). This is particularly important in the epidermis, which forms the protective outer barrier of the body (1, 4). Extracellular nucleotides

have also been recognized as important mediators of other physiological signals such as pain sensation (2, 10), keratinocyte proliferation, migration, and apoptosis, and activation of inflammatory cells (11–13). Besides via cellular lysis, nucleotides can also be released by exocytosis, or by diffusion through different plasma membrane channels (14–17). In the extracellular milieu UTP and ATP are rapidly degraded to corresponding diphosphates and monophosphates (4). As nucleotides are released at relatively high concentrations and metabolized rapidly, they are ideal signaling molecules in tissues (18).

Extracellular nucleotides signal via specific ligand-gated or G protein-coupled receptor families (P2X and P2Y, respectively). These receptors are expressed in keratinocytes in a differentiation-dependent manner, members of the P2Y² family are mainly found in the basal cell compartment, representing the proliferative cell population (reviewed in Ref. 13). UTP signals via P2Y₂ and P2Y₄, and UDP via P2Y₆ and P2Y₁₄ (reviewed in Ref. 19). All of the UTP/UDP receptors are expressed in keratinocytes, although the levels of P2Y₄ and P2Y₁₄ are probably low (4, 7, 11, 20). The P2Y₂, P2Y₄, and P2Y₆ subtypes are coupled to G_q/G₁₁ proteins and activate phospholipase C, thus inducing inositol 1,4,5-trisphosphate-mediated Ca²⁺ release from the ER, and activation of PKC, whereas the P2Y₁₄ receptors inhibit adenylyl cyclase activity via G_{i/o} proteins (reviewed in Ref. 19).

Hyaluronan is a linear, high molecular mass polysaccharide composed of glucuronic acid and *N*-acetylglucosamine. The hyaluronan chain is built at the inner surface of the plasma membrane by hyaluronan synthase (*HAS*) enzymes, which also form a pore for hyaluronan transport into the extracellular space (21). The newly synthesized hyaluronan chain remains associated with the pericellular matrix, either bound to the synthase, or plasma membrane receptors like CD44, but is later released into the extracellular matrix, where it is often associ-

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² The abbreviations used are: P2Y, G protein-coupled purinergic receptor; qRT, quantitative RT; HAS, hyaluronan synthase; HYAL, hyaluronidase; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-GlcUA, UDP-glucuronic acid; UDP-Glc, UDP-glucose; PTX, pertussis toxin; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; p38, p38 mitogen-activated protein kinase; HaCaT, spontaneously transformed aneuploid immortal keratinocyte cell line from human skin; REK, rat epidermal keratinocytes; ER, endoplasmic reticulum; BIM, bisindolylmaleimide; EGFR, epidermal growth factor receptor; ELSA, enzyme-linked sorbent assay; DAB, diaminobenzidine; ANOVA, analysis of variance.

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ated with aggregating proteoglycans or other extracellular membrane molecules like IαI or TSG6 (reviewed in Ref. 22). There are three hyaluronan synthase enzymes in mammals (HAS1–3). The distribution of the isoenzymes in tissues and the regulation of their expression as well as their synthetic activity differ (23) allowing context-dependent regulation of hyaluronan synthesis.

Enhanced hyaluronan metabolism occurs with tissue injury and inflammation (reviewed in Refs. 24–26), situations that are also associated with extracellular release of nucleotides. However, practically nothing is known about the possible influence of these signaling molecules on hyaluronan synthesis. In gingival fibroblasts (27) and smooth muscle cells (28), *HAS1* expression is up-regulated by adenosine, a breakdown product of ATP, leading to the formation of hyaluronan-rich pericellular matrices. In human keratinocytes the sugar nucleotide UDP-glucose stimulates *HAS2* expression and hyaluronan synthesis (20).

In skin epidermis hyaluronan content is rapidly increased after tissue wounding (29), exposure to chemical irritants (30), and exposure to ultraviolet B radiation (UVB) (31). Elevations of *HAS2* and *HAS3* mRNA are seen after skin wounding (29, 32), and UVB radiation also induces *HAS1* (31). The mechanisms of *HAS* up-regulation after trauma remain unresolved at the moment, although activation of the EGF family growth factors by an insult may at least partly explain the up-regulation of *HAS2* and *HAS3* (32, 33).

The present work explores the hypothesis that the extracellular nucleotide UTP and its breakdown products UDP and UMP contribute to the rapid *HAS* expression and hyaluronan accumulation after various skin traumas. We establish for the first time that extracellular UTP causes a pulse of hyaluronan synthesis via a strong, specific and rapid up-regulation of *HAS2* expression, mediated by the activation of p38, ERK, STAT3, CaMKII and CREB, whereas the expressions of *HAS1*, *HAS3*, *HYAL1*, and *HYAL2* are unaffected. High concentrations of UDP reproduce the effect, whereas UMP had no significant influence on *HAS2* expression.

Results

Extracellular UTP Enhances Hyaluronan Production—The influence of UTP on hyaluronan metabolism of human keratinocytes was studied by treating HaCaT cells with 100 μM UTP and analyzing hyaluronan staining of the cultures and the amount of hyaluronan secreted in the growth medium. The staining intensity was clearly higher in the UTP-treated cultures compared with the untreated cultures already after a 2-h exposure (Fig. 1, A and B). Confocal imaging confirmed the accumulation of hyaluronan (Fig. 1, C and D) and showed its localization close to the plasma membranes as seen in the side views of the cell layers (Fig. 1, E and F). The histochemical stainings indicated an early activation of its synthesis. However, the amount of hyaluronan released into the culture medium was just slightly increased after a 4-h incubation, the more substantial increase requiring a 6-h incubation with UTP (Fig. 1, G and H). At that time point the amount of hyaluronan in the culture medium was increased by 24 and 46% in the cultures treated with 10 and 100 μM UTP, respectively (Fig. 1, G and H).

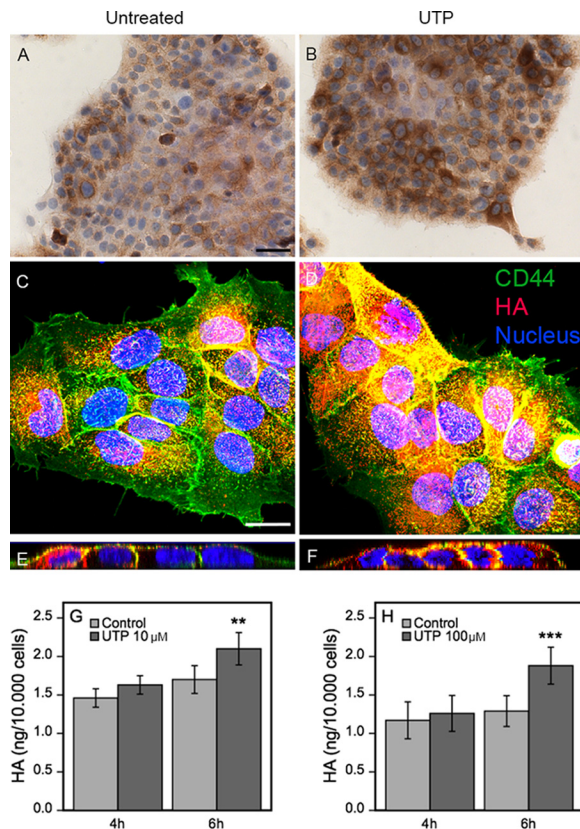


FIGURE 1. UTP addition in keratinocyte cultures rapidly increases pericellular hyaluronan, and induces a subsequent release of hyaluronan into the medium. HaCaT cultures were left untreated (A, C, and E) or treated with 100 μM UTP for 2 (B) and 4 h (D and F) and stained for hyaluronan using bHABC. In A and B, DAB was used as a chromogen (brown color). In C–F, the cultures were stained for hyaluronan using bHABC and TR-streptavidin (red), and for CD44 with a FITC-labeled secondary antibody (green). The nuclei were visualized with DAPI (blue). C and D are compressed stacks of the confocal images, E and F are side views cut through such stacks. The magnification bar for the bright field images is 50 μm , and for confocal images 20 μm . Culture media collected from HaCaT cells treated with 10 μM for 4 and 6 h ($n = 3$ for both) (G) and 100 μM (H) UTP for 4 and 6 h ($n = 4$ and $n = 9$, respectively) were analyzed for hyaluronan secretion. The data represent mean \pm S.E. Mixed model ANOVA was used to calculate the significance of the difference to untreated cultures (**, $p < 0.01$; ***, $p < 0.001$).

UTP and UDP Markedly Up-regulate *HAS2* Expression—To explore the cause of the increased hyaluronan secretion induced by UTP we first analyzed the possible influence of UTP on the level of the hyaluronan precursor sugars, UDP-GlcNAc and UDP-GlcUA, known to control the rate of hyaluronan synthesis (34–40). No significant changes in their levels were, however, observed in the UTP-treated cells compared with untreated cultures (Fig. 2A), excluding their contribution to hyaluronan accumulation. Similarly, addition of UTP to the culture medium did not influence the amount of intracellular UTP (Fig. 2B).

We then screened the expression levels of the hyaluronan-related genes by qRT-PCR at the 2-h time point (Fig. 2, C and D). *HAS2* mRNA levels in the HaCaT cultures subjected to 100 μM UTP were markedly elevated, with a mean 9.2-fold increase (range 4–35-fold, $n = 15$) (Fig. 2C). UTP up-regulated *HAS3* expression in some of the experiments, but the fold-change was more modest than for *HAS2*, and not statistically significant (Fig. 2D). The mRNA levels of *HAS1*, *HYAL1*, and *HYAL2* were not influenced by UTP (Fig. 2D).

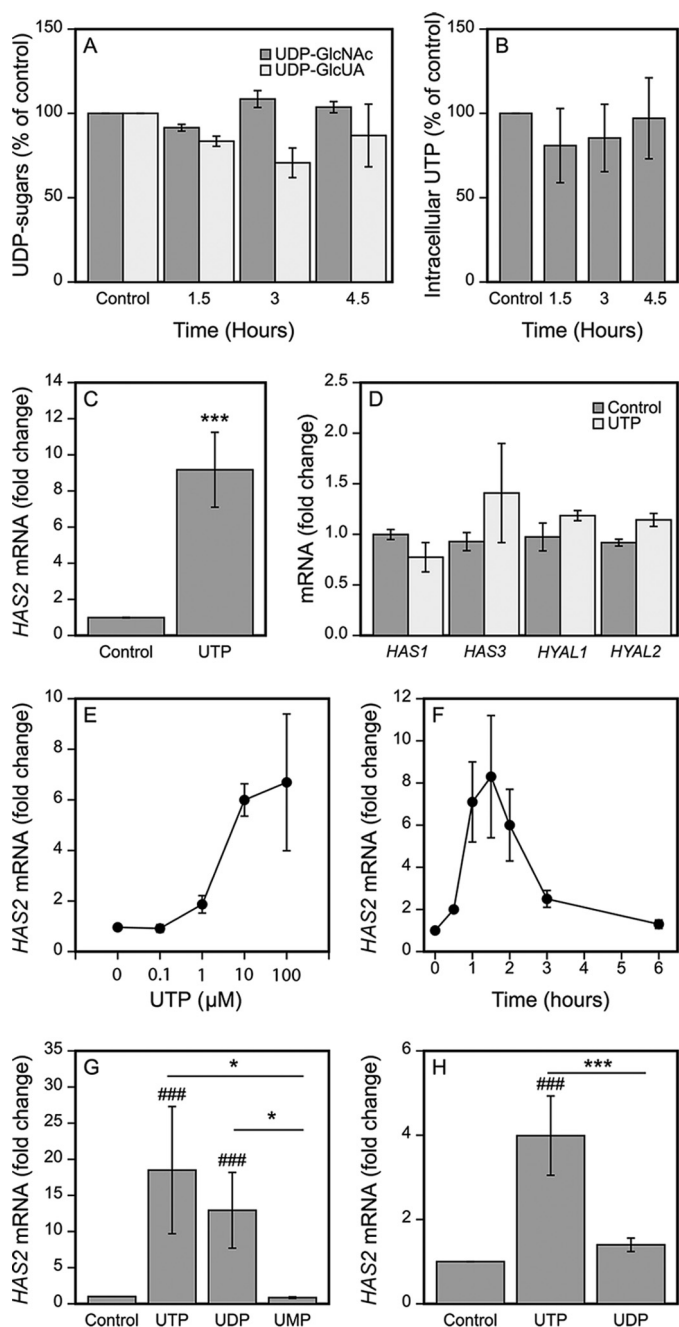


FIGURE 2. UTP strongly up-regulates *HAS2* expression. HaCaT cells were incubated for the indicated times with 100 μM UTP, the amounts of the intracellular UDP-sugar precursors of hyaluronan were measured (A) as well as the intracellular UTP (B). In panels C and D, HaCaT cells were incubated for 2 h with 100 μM UTP and the levels of *HAS2* mRNA (C, $n = 15$) and other hyaluronan-related genes (D, *HAS3*, $n = 4$; others $n = 3$) were analyzed by qRT-PCR. E, HaCaT cells were treated with 0.1–100 μM UTP for 2 h ($n = 3$). F, 100 μM UTP was added to the cultures and the samples were collected after different incubation times for *HAS2* mRNA assays ($n = 3$). G, HaCaT cells were treated for 2 h with 100 μM UTP, UDP, and UMP prior to qRT-PCR analysis ($n = 3$). H, HaCaT cells were treated for 2 h with 10 μM UTP and UDP prior to mRNA analysis ($n = 3$). Statistical significances of the differences between the groups were tested using (in C and D) one group t test (***, $p < 0.001$). In G and H, mixed model ANOVA was used for comparisons between the different treatments (indicated by *, $p < 0.05$) and comparisons of treatments to controls (set to 1) using pnorm (indicated by ###, $p < 0.001$). For the UDP-sugars (A) and concentration and time series (E and F) the non-parametric Friedman test was used due to unequal variances between the groups. Both the effects of the UTP concentration (E) and of the incubation time (F) were statistically significant ($\chi^2 = 11.46$, Friedman test $p = 0.022$ and $\chi^2 = 16.7$, Friedman test

Different doses of UTP applied into the culture medium showed that the maximum response to UTP was obtained at about 10 μM , whereas a 1 μM concentration induced just about 2-fold stimulation in *HAS2* expression (Fig. 2E). The concentration of UTP needed to stimulate *HAS2* expression exceeded that present under basal conditions (nanomolar range), but in stimulated keratinocytes UTP is released at micromolar concentrations (41). Under pathological conditions the concentration of ATP can reach even at 700 μM in the tumor microenvironment (42). Under these conditions the concentration of UTP is also high, as it is released at a 1:3–1:5 ratio to ATP in several cell types both under basal and mechanically stimulated conditions (41).

The level of *HAS2* mRNA started to rise already at 30 min after adding UTP, reaching its maximum at 1.5 h (Fig. 2F). Three hours after introduction of UTP the *HAS2* mRNA rise had largely faded, and completely disappeared at the end of the 6-h follow-up (Fig. 2F).

100 μM UDP exhibited a comparable stimulatory effect on *HAS2* expression as 100 μM UTP (Fig. 2G). However, the effect of 10 μM UDP was markedly smaller compared with 10 μM UTP (Fig. 2H). In contrast to UTP and UDP, 100 μM of the monophosphate UMP tended to down-regulate *HAS2* expression although the response did not reach statistical significance (0.6-fold) (Fig. 2G).

Induction of *HAS2* Expression by UTP Involves the Purinergic $P2Y_2$ Receptor—UTP is known to signal via the G-protein-associated receptors $P2Y_2$ and $P2Y_4$, whereas UDP utilizes $P2Y_6$ and $P2Y_{14}$ (43). Although it has been reported that all of these receptors are expressed in human keratinocytes, the expression levels of $P2Y_4$ and $P2Y_{14}$ seem to be low (4, 7, 11, 44).

To reveal the role of $P2Y_2$, we suppressed its expression by specific siRNAs. The efficiency of the siRNA silencing of *P2Y2* mRNA was 59% (Fig. 3A). The reduction of *P2Y2* did not influence *HAS2* expression under the basal culture conditions, but it suppressed the UTP-induced *HAS2* up-regulation in all experiments performed, the mean reduction being 62% (Fig. 3B). As UTP may experience a rapid extracellular degradation to UDP in the culture medium, we explored the possible involvement of the UDP receptors in the UTP response. At first we silenced the $P2Y_6$ receptor with specific siRNAs. Although the efficiency of the silencing was 59% (mean of 5 independent experiments), its influence on the UTP-induced rise in *HAS2* was variable, and statistically not significant (data not shown). To further explore the role of the $P2Y_6$ receptor we utilized a selective antagonist of $P2Y_6$, MRS2578. At a concentration of 20 μM this inhibitor was able to reduce the UDP-induced *HAS2* up-regulation by 34% ($p = 0.084$, Fig. 3C). It also tended to slightly (13%) reduce the UTP-induced *HAS2* response, however, these effects did not reach statistical significance (Fig. 3D). The other UDP receptor, $P2Y_{14}$, is coupled with the G_i protein, which is inhibited by pertussis toxin (PTX) (45). We have previously shown that PTX treatment is effective in HaCaT cells in blocking a UDP-Glc-induced *HAS2* induction without causing toxic

$p = 0.01$, respectively). No significance was found in UDP-GlcNAc and UDP-GlcUA (A) ($\chi^2 = 4.667$, $p = 0.097$, and $\chi^2 = 5.0$, $p = 0.172$, respectively) between the untreated and UTP-treated cultures.

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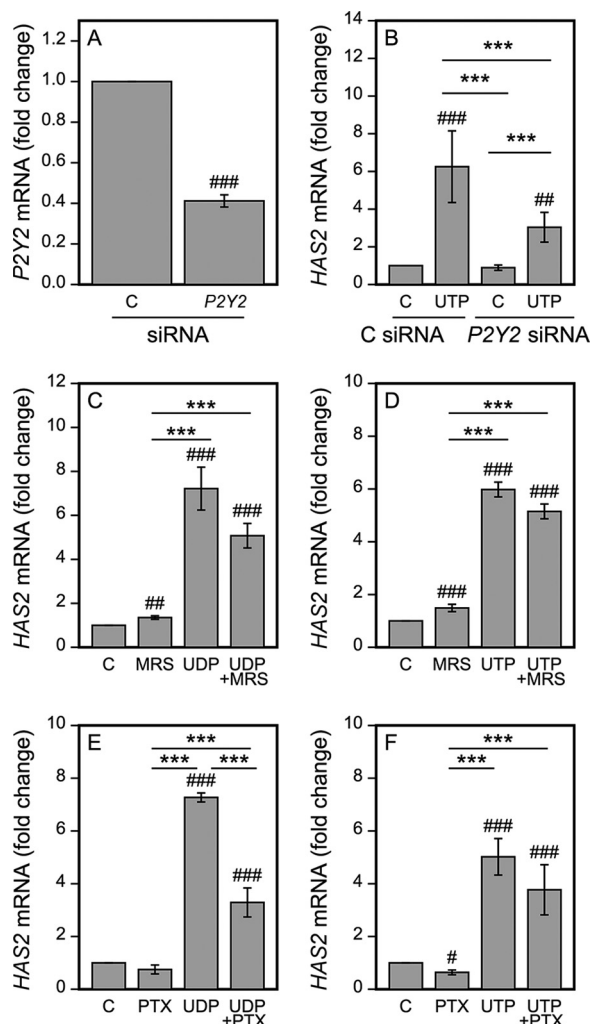


FIGURE 3. The P2Y receptors and signaling pathways involved in the UTP-induced *HAS2* up-regulation. HaCaT cells were transfected with control and P2Y₂-specific siRNAs (A and B). A, 2 days after the transfection the samples were collected to test for the efficiency of the siRNAs ($n = 4$), or B, subjected to 100 μM UTP for 2 h prior to collecting the samples for *HAS2* qRT-PCR ($n = 5$). C and D, cells were subjected to MRS2578 (a selective antagonist of P2Y₆, 20 μM) for 30 min, and E and F, PTX (100 ng/ml) for 17 h prior to the addition of 100 μM UDP (C and E) and UTP (D and F) for 2 h before mRNA assays. Statistical significances of the differences between the groups were tested using one group t test in A (##, $p < 0.01$). Mixed model ANOVA was used for comparisons between the different treatments (indicated by **, $p < 0.01$; ***, $p < 0.001$) and comparisons of treatments to controls (set to 1) was tested by p norm (indicated by #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$) in B–F.

effects (20). Here, we found that PTX pretreatment reduced the UDP-induced *HAS2* up-regulation by 64% (Fig. 3E), but was unable to consistently block the UTP-induced up-regulation of *HAS2* (Fig. 3F). These data suggest that the UTP-induced *HAS2* response is conveyed mainly through the UTP receptors.

UTP Rapidly Activates p38, ERK, STAT3, CaMKII, and CREB—The UTP signal mediated by the G_{q/11} protein coupled to P2Y₂, P2Y₄, and P2Y₆ involves activation of calcium-related pathways, eventually leading to phosphorylation of MAP kinases, calmodulin, and the associated kinases such as CaMKII (19), STATs (46), and CREB (47). To study whether these pathways are also involved in the *HAS2* response we performed Western blotting experiments on the corresponding phospho-proteins. Although UTP did not influence the amount of total

protein of these signaling molecules, it caused a clear and rapid activation of phospho-p38 (Fig. 4A), pERK (Fig. 4B), and pCREB (Fig. 4C). A tendency for enhanced phosphorylation by UTP was also found at Ser-727 of STAT3 with a similar temporal pattern as in p38, ERK, and CREB (Fig. 4D), whereas there was no influence on the Tyr-705 phosphorylation of STAT3 (Fig. 4D).

Staining with the pCaMKII antibody was positive under the basal culture conditions of HaCaT cells, localizing mainly in the nucleus (Fig. 4E). After a 30-min treatment with UTP the intensity of nuclear staining was further increased (Fig. 4, F and G), indicating CaMKII activation.

The UTP-induced *HAS2* Up-regulation Is Reduced by the Inhibition of PKC, p38, ERK, CaMKII, STAT3, and CREB—To obtain further support for the involvement of the above UTP-activated signaling pathways in the *HAS2* response, we pretreated the HaCaT cultures with inhibitors of the corresponding proteins prior to UTP exposure. Treatment with the p38 inhibitor BIRB796 reduced the UTP-induced *HAS2* up-regulation by 52%, whereas it had no influence on the basal level of *HAS2* (Fig. 5A). Similarly, the MEK/ERK inhibitor PD98059 cut the UTP-induced *HAS2* expression by 48%. However, it also significantly reduced the basal expression of *HAS2* by 30% (Fig. 5A). As MAPKs can be activated by PKC, we treated the HaCaT cells with the PKC inhibitor BIM (Fig. 5B). It also reduced the UTP-induced *HAS2* expression by 48% (Fig. 5B).

Inhibition of CaMKII signaling with KN93 suppressed the UTP-induced *HAS2* rise by 80% without influencing the basal expression level (Fig. 5C). Likewise, the STAT3 inhibitor IX (CPD188) exerted an 87% cut in the UTP-induced response without influencing the basal *HAS2* expression (Fig. 5D). KG501 (naphthol-AS-E-phosphate), which blocks the binding of CPB to CREB and thereby prevents pCREB activity, reduced the *HAS2* mRNA induction due to UTP in all the experiments performed (39% inhibition) (Fig. 5E). Its analog, naphthol-AS-BI-phosphate (48), caused a 31% reduction in the UTP-induced *HAS2* stimulation ($p < 0.001$, $n = 4$, data not shown).

As G-protein signaling is also known to activate EGFR (reviewed by Ref. 49) and JAK2 (20, 50) we pretreated the UTP-exposed cultures with AG490, which inhibits both EGFR and JAK2. However, here it failed to block the UTP-induced *HAS2* response (Fig. 5F), indicating that the rapid *HAS2* response after UTP is independent of EGFR and JAK2.

Discussion

The present study shows for the first time that the extracellular uridine nucleotides UTP and UDP strongly up-regulate *HAS2* expression, leading to the accumulation of hyaluronan in the pericellular matrix and later in the culture medium. As these nucleotides are often released after tissue trauma, the present findings provide a novel signaling mechanism to explain the rapid increase in hyaluronan synthesis detected after various insults (29–32). It also links hyaluronan to the defense mechanisms activated by danger signals such as extracellular nucleotides. The UTP-induced increase in *HAS2* activation is transient, however, indicating that a more sustained response observed after wounding and UVR (29, 31, 32)

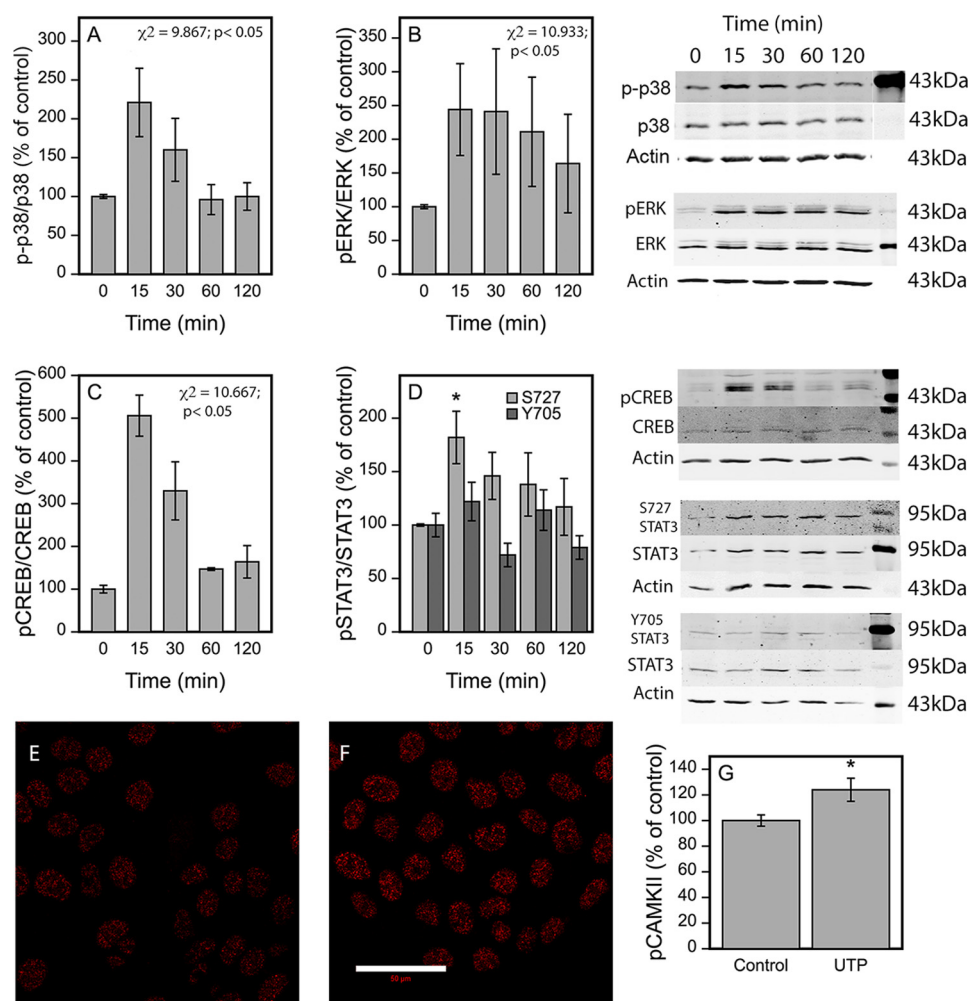


FIGURE 4. UTP induces rapid phosphorylations of p38, CREB, and Ser-727 of STAT3. HaCaT cells were subjected to 100 μM UTP for 15 min to 2 h before collecting the samples for Western blotting using antibodies against phosphorylated signaling proteins. For normalization the blots were reprobed for the total forms of signaling proteins and actin after a brief (10 min) stripping with 0.2 M NaOH. Representative blots out of 3 (p-p38, p38 α (A); pERK, ERK (B); pCREB, CREB (C); pSTAT3-S727, pSTAT3-Y705, and total STAT3 (D)) experiments with the corresponding actins are shown, whereas mean \pm S.E. of the quantifications of the phosphorylated forms per corresponding total proteins from all the experiments are presented as bar graphs. Statistical significances were tested using a non-parametric Friedman test because of unequal variances, indicating the overall significance values indicated in the figures. For STAT3-Y705 and -S727 the overall influence did not reach statistical significance. For Ser-727 of STAT3, allowing the use of a parametric test, pairwise comparisons of individual time points to the zero time point utilizing mixed model ANOVA and pnorm function were also performed, suggesting a significant influence at the 15-min time point (indicated by *, $p < 0.05$) (D). E and F, immunostainings for pCAMKII of the untreated (E) and the UTP-treated (100 μM) (F) cultures. The treatment time was 30 min. Mean \pm S.E. of 5 experiments are shown in the chart (G). Statistical significance between the groups was tested using mixed model ANOVA (indicated by *, $p < 0.05$) (G).

requires subsequent activation of other signaling routes, such as growth factors or cytokines (33, 51, 52).

UTP Selectively Activates HAS2 in Keratinocytes—Although HAS2 underwent a marked increase, the effect of UTP on HAS3 was slight and inconsistent, and completely non-existent on HAS1. The absence of influence on HYAL1 and HYAL2 and the unchanged levels of the UDP-sugar substrates also support the idea that the accumulation of hyaluronan was specifically due to HAS2 up-regulation. A similar HAS2-specific response of keratinocytes was previously found for the growth factors EGF and KGF (33, 51, 52), whereas HAS2 and HAS3 contribute to keratinocyte hyaluronan production at approximately equal proportions under basal culture conditions (31). In addition to direct transcriptional regulation of HAS2, the effect of UTP could arise from the induction of HAS2-AS1, which in turn can regulate HAS2 expression via chromatin remodeling (53).

Although the UTP-induced HAS2 induction was rapid and transient, it was associated with a significant increase in the amount of hyaluronan in the pericellular matrix and in the culture medium, indicating that the elevated mRNA levels were reflected in HAS2 protein activity. The possible importance of producing hyaluronan specifically by HAS2 is not known, but it has been found that the appearance of the cell surface coat produced by overexpressed HAS2 is slightly different from that made by HAS3 (54). Although the increase in hyaluronan followed the increase in HAS2 mRNA, it is possible that post-translational modifications such as phosphorylation, ubiquitination, or O-GlcNAcylation of HAS2 were involved in the UTP-induced hyaluronan synthesis (55, 56, 39). However, the involvement of O-GlcNAcylation is less likely, because UTP did not influence the level of UDP-GlcNAc, an important substrate for O-GlcNAcylation.

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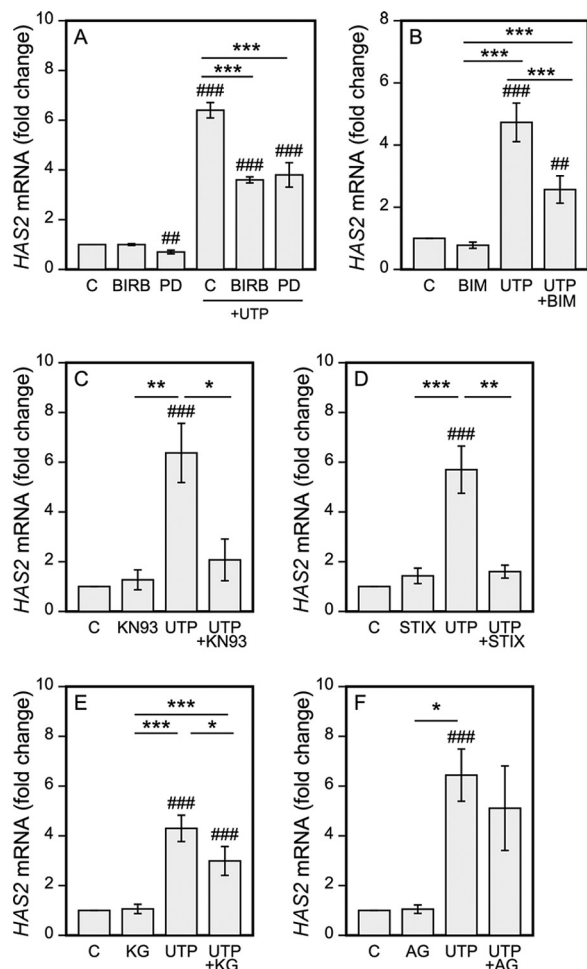


FIGURE 5. Inhibition of p38, CaMKII, STAT3, and CREB reduce the UTP-induced *HAS2* up-regulation. HaCaT cells were subjected to: *A*, the MEK kinase inhibitor PD98059 (PD, 0.5 μ M) and the p38 inhibitor BIRB796 (BIRB, 2 μ M); *B*, the PKC inhibitor (BIM, 10 μ M); *C*, the CaMKII inhibitor KN93 (KN93, 25 μ M); *D*, the STAT3 IX inhibitor (STIX, 50 μ M); *E*, the CREB inhibitor KG501 (KG, 25 μ M); and *F*, the JAK2/EGFR inhibitor AG490 (AG, 30 μ M). Preincubations with the inhibitors were 0.5 h for ERK, p38, CREB, and PKC, and 2 h for CaMKII and STAT3 prior to the addition of 100 μ M UTP for 2 h. Mean \pm S.E. are shown. The number of experiments is 3 for ERK and p38, 6 for BIM, 4 for CREB, 6 for CaMKII, 5 for STAT3, and 4 for JAK2/EGFR. Statistical significances of the differences between the groups were tested using mixed model ANOVA (indicated by *, **, and ***) and comparisons of the treatments to the controls (set to 1, indicated by ## and ###) using pnorm. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The increase of hyaluronan surrounding the cells was detectable within 2 to 4 h, whereas reaching a significant change in the released hyaluronan required 6 h, probably due to the relatively long half-life (8 h) of pericellular hyaluronan in keratinocytes (57). Treatment of mesenchymal cells with a viral mimetic, or subjecting them to ER stress or hyperglycemia leads to the formation of cable-like structures of hyaluronan that attract inflammatory cells (26). Although not as prominent as in mesenchymal cells, keratinocytes can also form monocyte adhesive hyaluronan cables in response to inflammatory cytokines (58). UTP appears to differ from the above mentioned conditions in this respect, as no signs of cable formation were observed.

Receptors Involved in the UTP-induced *HAS2* Response—UTP signals via the P2Y₂ and P2Y₄ receptors, however, the expression level of P2Y₄ appears to be very low in keratinocytes

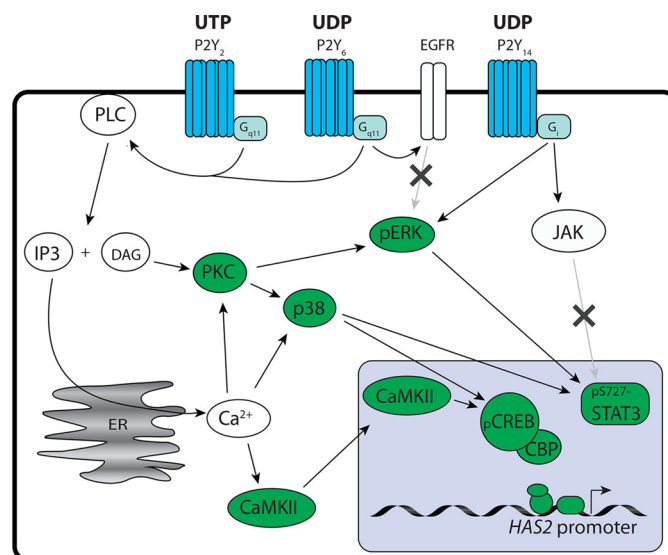


FIGURE 6. Schematic representation of the signaling pathways involved in the UTP-induced *HAS2* up-regulation. Extracellular UTP and its breakdown product UDP activate the P2Y receptors that increase *HAS2* expression via the indicated signaling steps. Those steps positively verified in the current study are marked green, whereas the pathways excluded are indicated by an “x.”

(4, 7, 11), suggesting that it has a minor role in the UTP-induced responses in these cells. On the other hand, a P2Y₂-specific siRNA reduced the UTP-induced *HAS2* response by 62%, which considering only a partial (59%) silencing of the mRNA expression, indicates that the P2Y₂ receptor mediates a major proportion of the effect.

As UTP released into the extracellular space is actively metabolized to UDP also by the HaCaT cells (4) and UDP enhanced *HAS2* mRNA expression, it was possible that a part of the *HAS2* induction after UTP release might occur via the P2Y₆ and P2Y₁₄ receptors for UDP (Fig. 6). Indeed, under our experimental conditions, inhibitors of P2Y₆ and P2Y₁₄, which reduced the UDP-induced *HAS2* response, caused a slight, although nonsignificant down-regulation of the UTP response. The higher concentration of UDP required to achieve a stimulation comparable with that by UTP, together with the modest effect of the UDP-receptor inhibitors on UTP-induced *HAS2* response, suggest that UTP degradation to UDP is not necessary for *HAS2* up-regulation, although it may contribute to the response. In contrast to UTP and UDP, the corresponding monophosphate UMP failed to induce *HAS2* expression. This might be explained by the fact that no known receptor for this nucleotide exists. The finding also suggests that no significant reversal of extracellular UMP to UDP or UTP occurs in the HaCaT cell cultures.

The UTP-induced Activation of *HAS2* Expression Is Mediated via Pathways Dependent on CaMKII and PKC—As P2Y₂, P2Y₄, and P2Y₆ are associated with the same G-protein, G_{q/11}, it is likely that their downstream signaling is similar, involving intracellular Ca²⁺ release and phospholipase C. MAP kinases, calmodulin, and related kinases as well as the transcription factors STAT3 and CREB are known to be activated by these receptors, reviewed in Ref. 13 and outlined in Fig. 6. UDP, on the other hand, via its association with P2Y₁₄ and its inhibitory

partner G_i , can suppress cAMP formation thereby reducing CREB activity. However, $P2Y_{14}$ can also activate STAT3 via ERK and JAK2 (20, 59). In general, our findings on the activation of MAPK, CaMKII, STAT3, and CREB in HaCaT cells by UTP stimulation are in line with previous reports. The experiments applying specific inhibitors of these signaling molecules showed markedly reduced *HAS2* responses to UTP, indicating involvement of PKC, p38, ERK, CaMKII, STAT3, and CREB. However, the true role of pERK in the *HAS2* response remains uncertain due to the strong effect of the inhibitor on the basal *HAS2* expression level. In any case, co-activation of both ERK and p38 signaling has been demonstrated in HaCaT cells after nucleotide exposure (60). The inability of the JAK2/EGFR inhibitor AG490 to block the UTP-dependent *HAS2* response rules out EGFR activation after extracellular nucleotide exposure (61).

Although CREB is a target of several of the indicated upstream signaling pathways, STAT3 is regulated by MAP kinases and JAK2 (62). p38 and JNK have been associated with the Ser-727 phosphorylation of STAT3, whereas JAK2 is associated with Tyr-705 phosphorylation (reviewed in Ref. 63). UTP induced STAT3 phosphorylation in Ser-727, but not in Tyr-705, the former correlating temporally with p38 activation. These findings are in line with the inhibitor experiments indicating the involvement of p38 but not JAK2 in *HAS2* mRNA induction. Although the specific significance of Ser-727 phosphorylation is unsettled, it is needed for full transcriptional activation of STAT-regulated gene expression, for example, by recruiting co-activators to the promoter (63, 64). Although Tyr-705 phosphorylation has been classically regarded as necessary for STAT3 activity, recent findings suggest that STAT3 may also influence gene expression independently of its phosphorylation status (reviewed Ref. 63).

Blocking CREB binding to CBP, a necessary cofactor for CREB activity (65), and inhibition of STAT3 activity both reduced the UTP-induced *HAS2* up-regulation. Both of these transcription factors have active binding sites on the *HAS2* promoter and regulate *HAS2* expression in keratinocytes (66, 67). The lower efficiency of CREB blocking may be due to a lower potency of the inhibitors used, or a weaker activity of CREB on the *HAS2* promoter. CREB alone, even with CBP, has been suggested to be insufficient to activate transcription (65). However, as both STAT3 and CREB inhibitors influenced UTP-induced *HAS2* expression, it is also possible that their effect is cooperative as shown with some other promoters (68, 69).

The Interplay between UTP and Hyaluronan—The rapid nature of the *HAS2* response suggests that it may be a part of the cellular defense reactions induced by UTP signaling. Indeed, the UTP-induced *HAS2* expression occurred with a similar temporal pattern as the influence of UTP on the expression of the proinflammatory cytokines IL-6 and IL-8 in HaCaT cells (7, 70). The simultaneous response rules out the possibility of the cytokines controlling *HAS2* expression or vice versa; rather, it is likely that the early *HAS2* and cytokine responses are coordinated. Later on, breakdown of hyaluronan to low molecular mass fragments can potentiate IL-6 signaling, resulting in further IL-6 production (71), whereas intact hyaluronan dampens the inflammatory reaction by counteracting the IL-6 signaling

(72–74). Apart from regulating cytokine release, hyaluronan itself can protect cells from apoptosis by scavenging reactive oxygen species. This has been shown in cultured corneal epithelial cells and keratinocytes treated with either UV radiation or toxic substances (75, 76). Furthermore, Wang and co-workers (77) showed that high levels of hyaluronan and *Has2* expression were associated with resistance to UVB radiation and serum starvation in mouse fibroblasts. Thus, tissue stress that liberates UTP is followed by a rapid build-up of a hyaluronan matrix, which might protect the cells from further damage in case the harmful insult continues.

HAS2 activation and hyaluronan may also have signaling functions in their own right. Via its cell surface receptor CD44, hyaluronan has been shown to modulate the signaling of several growth factor receptors like EGFR and PDGFR, as reviewed in Ref. 78, often potentiating the effects of the native ligands. Interestingly, in keratinocytes and fibroblasts, synergism was found in the mitogenic response between extracellular nucleotides and EGF, TGF α , PDGF, and TGF β (79). Further studies are needed to check whether hyaluronan is involved in this cooperation.

UTP stimulates the migration of endothelial cells, arterial smooth muscle cells, corneal epithelial cells, prostate cancer cells, and schwannoma cells (80–83), whereas in keratinocytes it inhibits cell spreading and motility (84, 85). The influence of hyaluronan on cell motility is obviously complex. It has been shown to stimulate cell migration and in turn its removal inhibits migration (reviewed in Ref. 86). However, hyaluronan can also inhibit cell motility, especially when present in excessive amounts such as during artificial overexpression induced by *HAS* transfection (87, 88). The physiological response of the cells to hyaluronan depends on its molecular mass (24, 89). UTP induced mainly *HAS2*, which is known to synthesize high molecular mass hyaluronan (87, 90). Whether hyaluronan breakdown was activated by UTP was not investigated here. However, we did not observe any increase in the intracellular hyaluronan, which is often associated with hyaluronan breakdown (31, 91). Moreover, the expression of hyaluronidases was not changed. However, because the inhibition of cell motility occurs already within 15 to 30 min after the UTP exposure (84, 85), it unlikely relates to hyaluronan synthesis, which takes place later.

Coordination and Convergence of Signaling in Keratinocyte Hyaluronan Responses Triggered by Injury and Environmental Stress—Interestingly, the UTP-induced signaling pathways regulating *HAS2* expression partially overlap with those occurring after UVB exposure, an insult known to cause nucleotide release in keratinocytes (9). Although UVB induces a multitude of signaling events, in rat epidermal keratinocytes (REK) the induction of *Has2* and *Has3* appears to depend mainly on p38 and CaMKII (31). However, in REK cells UVB also activates the expression of other hyaluronan-related genes, such as *Has1*, *Hyal1*, and *Hyal2* (31), which did not respond to UTP. Furthermore, the UVB-induced activation of p38 and *Has2* expression in rat keratinocytes is long-lasting (36 h), suggesting that for a more sustained response other signaling pathways need to be activated. One of the UVB-activated cascades originates from HB-EGF/EGFR (92), shown to regulate *Has2* and *Has3* expres-

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sion in REK cultures (32, 93). Although the intracellular signaling mediators triggered by nucleotides and EGFR activation are partially different, they show convergence, suggesting that they can act synergistically (94).

In conclusion, nucleotides UTP and UDP, but not UMP, released into the extracellular space activate *HAS2* mRNA expression in human keratinocytes. The expression of the other hyaluronan synthases or hyaluronidases are not significantly influenced by UTP. The signaling pathway for UTP involves the purinergic P2Y₂ receptor, and to a smaller extent the UDP receptors P2Y₆ and P2Y₁₄, and their downstream cascades recruiting PKC, and the MAP kinases p38 and ERK, CaMKII, STAT3, and CREB (Fig. 6). The effect of UTP on *HAS2* expression is strong and, although transient, causes a significant increase in hyaluronan accumulation in the pericellular matrix and culture medium. This rapid induction of a hyaluronan coat may be a good way to provide an instant response to a potentially damaging signal, whereas making sure a stronger, more sustained response is only activated if required by the circumstances.

Experimental Procedures

Cell Culture—HaCaT cells, a human spontaneously immortalized epidermal keratinocyte cell line developed by Boukamp *et al.* (95), were used in the present study. They were cultured in DMEM with low glucose (D5921, Sigma) containing 10% FBS (HyClone, Logan, UT), 2 mM L-glutamine (Euroclone, Milan, Italy), 50 units/ml of penicillin, and 50 μg/ml of streptomycin (Euroclone). For microscopy the cells were plated and cultured on 8-well chamber slides (Nunc Nalgene, Naperville, IL), and for biochemical experiments on 12- or 6-well plates (Greiner Bio-One, Kremmsmünster, Austria). UTP, UDP, and UMP (Sigma) were dissolved in H₂O as stock solutions and kept at −20 °C until used.

Signaling Inhibitors—A 2-h pretreatment before the addition of nucleotides was used with the CaMKII inhibitor KN93 (25 μM, Calbiochem, Merck Millipore, Darmstadt, Germany), JAK2 inhibitor AG490 (30 μM, Sigma), STAT3 inhibitor IX (50 μM, Calbiochem), and a 30-min pretreatment with the MEK inhibitor PD98059 (0.5 μM, Calbiochem), p38 inhibitor BIRB796 (2 μM, Axon Medchem BV, Groningen, Netherlands), CREB inhibitors (KG501, Sigma, and naphthol AS-BI-phosphate, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, both at 25 μM concentration), PKC inhibitor BIM (bisindolylmaleimide, 10 μM, Calbiochem), and the P2Y₆ inhibitor MRS2578 (20 μM, Tocris Biosciences, Southampton, UK), whereas with PTX (100 ng/ml, Sigma) a 17-h preincubation was used. KN93, PTX, BIM, and naphthol AS-BI-phosphate were dissolved in water, AG490 in ethanol, and the STAT3 inhibitor IX, BIRB796, KG501, and MRS2578 in DMSO. Equal amounts of these solvents were added to the control cultures.

siRNA Treatments—The control siRNAs were obtained from Eurogentec (Liege, Belgium), P2Y₂-targeted siRNAs were from Thermo Fisher (Waltham, MA), and P2Y₆-targeted siRNAs from Origene (Rockville, MD). Subconfluent cultures were transfected with 50 nM siRNA using RNAiMAX (Invitrogen) according to the manufacturer's instructions. The transfection medium was replaced with ordinary culture medium after 4 h.

TABLE 1
Primer sequences for qRT-PCR of the human genes

Gene name	Primer sequence (5' to 3')	T _m
<i>ARPO</i>	Forward, AGATGCAGCAGATCCGCAT	59
	Reverse, GTGGTGATACCTAAAGCCTG	
<i>HAS1</i>	Forward, CAAGATTCCTCAGTCTGGAC	59
	Reverse, TAAGAACCAGGAGAAAGCAG	
<i>HAS2</i>	Forward, CAGAATCCAAACAGACAGTTC	59
	Reverse, TAAGGTGTTGTGTGACTG	
<i>HAS3</i>	Forward, CTTAAGGGTTGCTTGCTTGC	59
	Reverse, GTTCGTGGGAGATGAAGGAA	
<i>HYAL1</i>	Forward, GCCCTCTATCCAGCATCTA	59
	Reverse, CTCACCCAGAGCACCCTC	
<i>HYAL2</i>	Forward, CCTCTGGGGCTTCTACCTCT	59
	Reverse, CTGAACACCGAAGCTCACA	
<i>P2Y2</i>	Forward, CCTGAGAGGAGAAGCGCAG	59
	Reverse, GAACTCTGCGGGAAACAGGA	

The cells were grown for 2 days before treatment with the nucleotides. The efficacy of the knockdown was confirmed by qRT-PCR.

RNA Extraction and qRT-PCR—Total RNA was extracted with the TRI Reagent (TR118, Molecular Research Center Inc., Cincinnati, OH) and cDNA synthesis was performed using the Verso cDNA kit (Thermo Fisher). The samples were analyzed on an MX3000P thermal cycler (Stratagene, La Jolla, CA), using the Fast Start universal SYBR Green Master Mix (ROX) (Roche Applied Science, Basel, Switzerland). The cycling conditions were as follows: preincubation for 10 min at 95 °C followed by 40 cycles of 15 s denaturation at 95 °C, 1 min annealing at a primer-specific temperature, and 1 min elongation at 72 °C. Gene-specific amplification was confirmed by a melt curve analysis. The specific primers for the genes analyzed and the annealing temperatures are shown in Table 1. Fold-inductions were calculated using the formula $2^{-(\Delta\Delta C_t)}$, where $\Delta\Delta C_t$ is $\Delta C_t(\text{sample}) - \Delta C_t(\text{non-treated replicate})$, ΔC_t is $C_t(\text{gene of interest}) - C_t(\text{ARPO})$ and C_t is the cycle at which the threshold is crossed.

Western Blotting—Protein extraction and SDS-PAGE were performed as described before (20). Briefly, RIPA (PBS, pH 7.4, with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml of phenylmethylsulfonyl fluoride, 10 μg/ml of sodium orthovanadate, and 1% phosphatase inhibitor mixture 2, and 0.5% protease inhibitor mixture (Sigma)) lysis buffer was used for protein extraction. 7.5–20 μg of protein was loaded per lane (based on Bradford assay), resolved by 10% SDS-PAGE, followed by transfer onto a nitrocellulose membrane (Amersham Biosciences™ Protran™ 0.45-μm NC, GE Healthcare Life Sciences, Little Chalfont, UK) with a Fastblot B43 semidry blotter (Biometra, GmbH, Göttingen, Germany). The membranes were washed with TBS containing 0.1% Tween, blocked with 1–5% BSA/TBS, and incubated with the primary antibodies: total STAT3, p38α, ERK1/2, CREB, and phospho-STAT3 (Tyr-705), phospho-STAT3 (Ser-727), phospho-CREB (Ser-133), phospho-p38 (all from Cell Signaling Technologies, Danvers, MA, diluted 1:1000), phospho-ERK (Santa Cruz Biotechnology Inc., CA, diluted 1:200), and followed by DyLight 680 or 800 anti-rabbit or anti-mouse secondary antibodies, 1:4000 (Pierce). Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) was used to image and quantitate the bands, normalizing the phosphospecific band intensities to the nonphosphorylated total proteins.

Hyaluronan ELSA—For hyaluronan assays HaCaT cells were treated with the nucleotide sugars (100 μM) for 4 or 6 h. The media were collected for hyaluronan assays and cells were released with trypsin and counted for normalization. The enzyme-linked sorbent assay (ELSA) of hyaluronan was performed as described earlier (96) using a probe complex containing the aggrecan G₁ domain and link protein (HABC, prepared as described in Ref. 97). ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with HABC, followed by 1-h sequential incubations with hyaluronan standards (1–50 ng/ml) and appropriately diluted samples, then by biotinylated HABC (bHABC) and horseradish peroxidase-streptavidin (Vector Laboratories, Burlingame, CA). A 3,3',5,5'-tetramethylbenzidine substrate was used for color development (0.01% 3,3',5,5'-tetramethylbenzidine, Sigma) in 0.005% H₂O₂, 0.1 M sodium acetate, and 1.5 mM citric acid buffer, pH 5.0. The reaction was stopped with 2 M H₂SO₄ and the absorbance read at 450 nm.

Hyaluronan and Immunostainings—The stainings were performed as described previously (57). Cells fixed in 2% paraformaldehyde were permeabilized and blocked with 1% BSA in 0.1 M phosphate buffer, pH 7.0, containing 0.1% Triton X-100, followed by overnight incubation at 4 °C with bHABC (3 $\mu\text{g}/\text{ml}$). The binding of bHABC was detected using the ABC complex (1:200, Vectastain Kit, Vector Laboratories) and 0.05% diaminobenzidine (DAB, Sigma) containing 0.03% hydrogen peroxide. The nuclei were counterstained with Mayer's hematoxylin. The stained cultures were viewed and imaged with a Zeiss Axio Imager.M2 microscope (Carl Zeiss Microimaging GmbH, Jena, Germany).

For dual stainings the anti-CD44 antibody Hermes 3 (a generous gift of professor Sirpa Jalkanen, University of Turku) at a 1:100 final dilution was added to the bHABC solution. Fluorescently tagged anti-mouse IgG (1:200) and Texas Red streptavidin (1:1000) (both from Vector Laboratories) were used as the secondary reagents. Nuclei were stained with DAPI (1 $\mu\text{g}/\text{ml}$, Sigma). The cells were viewed and imaged with confocal microscopy using a $\times 40$ NA 1.3 oil objective on a Zeiss Axio Observer inverted microscope, equipped with a Zeiss LSM 700 confocal module. The images were processed using ZEN 2009 software (Carl Zeiss), and Adobe Photoshop Elements 9 software (Adobe Systems Inc., San Jose, CA).

For the imaging of pCaMKII the cells were fixed in 4% paraformaldehyde for 20 min on ice, permeabilized with cold methanol for 10 min, followed by blocking with 1% BSA in Tris containing 0.1% Tween for 10 min at room temperature. Overnight incubation with a pCaMKII antibody (Promega, Madison, WI), diluted 1:500 in the blocking buffer, was followed by a 1-h incubation with a Texas Red-labeled anti-rabbit IgG antibody (1:500, Vector Laboratories).

UDP-Sugar Determination—For UDP-sugar determinations cells were treated with UTP (100 μM) for 1.5, 3, and 4.5 h after which the intracellular contents were extracted and UDP-GlcUA, UDP-GlcNAc, and nucleotides measured as described previously (54, 98). Briefly, the cells were washed twice with ice-cold PBS and scraped into PBS, followed by sonication and centrifugation (6000 $\times g$ for 20 min, all steps on ice or at 4 °C). A small aliquot was collected for total protein estimation with a

Pierce BCA kit (Thermo Fisher) and the rest of the sample was further purified with Superclean Envi-Carb SPE cartridges (Sigma). The eluted samples were evaporated and dissolved in water for HPLC on a CarpoPacTM PA1 column (Dionex, Thermo Fisher Scientific) as described before (98). Peak areas were normalized to the protein contents.

Statistical Methods—The results were analyzed with PASW Statistics 18 (SPSS Inc., Chicago, IL). Log transformation was used if the data were not normally distributed or the variances were unequal. Mixed model ANOVA and pairwise comparisons between the treatments were performed using the estimated marginal means (LSD) correcting for multiple comparisons. For comparisons between control (set as 1) and different treatments the cumulative distribution function (pnorm) calculated in R for Apple, version 3.2.3 (The R Project for Statistical Computing (99)), was used, correcting for multiple comparisons. For comparisons of a single group, normalized to a control group, the single group *t* test was used.

Author Contributions—T. J. conceived and designed the study, analyzed the results, and contributed to paper writing and prepared all figures. R. H. T. designed and coordinated the study, analyzed results, and wrote the paper. R. K. provided technical expertise for all experiments. G. B. took part in study conception. L. R., S. O., and S. P. S. performed and analyzed part of the experiments, and critically revised the manuscript. M. I. T. took part in the study conception and critically revised the manuscript. All authors approved the manuscript.

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