Bicarbonate is essential for protein tyrosine phosphatase 1B (PTP1B) oxidation and cellular signaling through EGF-triggered phosphorylation cascades

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Keywords: protein tyrosine phosphatase (PTP), thioredoxin reductase, peroxiredoxin, redox regulation, redox signaling, phosphorylation cascade, oxidative inactivation, peroxiredoxin system, peroxymonocarbonate, epidermal growth factor (EGF)

ABSTRACT

Protein tyrosine phosphatases (PTPs) counteract protein tyrosine phosphorylation and cooperate with receptor tyrosine kinases in the regulation of cell signaling. PTPs need to undergo oxidative inhibition for activation of cellular cascades of protein tyrosine kinase phosphorylation following growth factor stimulation. It has remained enigmatic how such oxidation can occur in the presence of potent cellular reducing systems. Here, using in vitro biochemical assays with purified, recombinant protein, along with experiments in the adenocarcinoma cell line A431, we discovered that bicarbonate, which reacts with H₂O₂ to form the more reactive peroxymonocarbonate, potently facilitates H₂O₂-mediated PTP1B inactivation in the presence of thioredoxin reductase 1 (TrxR1), thioredoxin 1 (Trx1), and peroxiredoxin 2 (Prx2) together with NADPH. The cellular experiments revealed that intracellular bicarbonate proportionally dictates total protein phosphotyrosine levels obtained after stimulation with epidermal growth factor (EGF) and that bicarbonate levels directly correlate with the extent of PTP1B oxidation. In fact, EGF-induced cellular oxidation of PTP1B was completely dependent on the presence of bicarbonate. These results provide a plausible mechanism for PTP inactivation during cell signaling and explain long-standing
Receptor tyrosine kinase (RTK) activation leads to transmission of downstream phosphorylation cascades upon growth factor stimulation, and has major importance in physiology and proliferative diseases such as cancer. Protein tyrosine phosphatases (PTPs), including PTP1B, counteract protein tyrosine phosphorylation and thereby act together with RTKs to regulate cell signaling (1). PTP activity depends upon a conserved active site Cys residue (1-4), which renders these enzymes susceptible to oxidative inactivation. This has a physiological importance, because RTK activation triggers transient H$_2$O$_2$ production from NADPH oxidases (NOXs) (5), which in turn leads to reversible oxidation and inhibition of PTPs. Inactivation of PTPs is believed to be absolutely required for RTK signaling (4,6). NOXs are membrane-localized (typically on the plasma, endosomal, or endoplasmic reticulum membrane) and use cytoplasmic NADPH to produce superoxide and H$_2$O$_2$ on the opposing membrane surface. The H$_2$O$_2$ must then enter the cell, facilitated through aquaporins (7,8), to exert its signaling actions. During stimulation of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptor pathways, the catalytic Cys residue of PTP1B becomes reversibly oxidized and thus inhibited (4,9). The initial oxidation product is a sulfenic acid (-SOH) (10,11), which can condense to an internal sulfenylamide (12-14) or undergo glutathionylation (15,16). Reversibly oxidized PTP1B can be reactivated by the thioredoxin (Trx) system (thioredoxin reductase 1 (TrxR1) and NADPH with or without Trx1 or thioredoxin-related protein (TRP14) (10,17-19), thus enabling the Trx system to modulate cellular RTK signaling (17).

It is much debated exactly how oxidation of PTP1B occurs in a cellular environment, especially considering that members of the peroxiredoxin (Prx) family of thiol proteins, which are expressed at high levels in cells and also recycled predominantly by the Trx/TrxR/NADPH system, are many orders of magnitude more reactive and therefore likely to react with nearly all intracellular H$_2$O$_2$ (20-22). Some cytosolic thiol-containing proteins can be oxidized via Prx-mediated relays (23,24), but this mechanism has not been demonstrated for PTP1B (25). With PTP1B being a therapeutic target with key roles in pathologies such as type 2 diabetes, obesity, and cancer (26,27), as well as an archetypical example of oxidative inactivation in redox signaling, it is important that the mechanism of this process becomes understood.

We recently characterized the effect of H$_2$O$_2$ on PTP1B activity and found that a reconstituted Prx2/Trx1/TrxR1/NADPH system, both through the removal of H$_2$O$_2$ and recycling of the oxidized PTP1B, prevent its inactivation (25). It thus remains unclear how PTP1B can be inactivated by H$_2$O$_2$ in the presence of a functional Prx/Trx-system. One possibility is that oxidation could be mediated by the more reactive peroxymonocarbonate (HCO$_4^-$), which is spontaneously formed in a reversible reaction between H$_2$O$_2$ and CO$_2$/bicarbonate (HCO$_3^-$) (28,29):

$$H_2O_2 + CO_2/HCO_3^- \rightleftharpoons HCO_4^- + H^+ / H_2O$$

The X-ray structure of peroxymonocarbonate shows a true hydroperoxide (30), and it has been structurally and kinetically characterized using $^{13}$C-NMR (28). Bicarbonate at physiological concentrations accelerates the reaction of H$_2$O$_2$ with thiols, thioethers, amines and other small molecules such as GSH and albumin via formation of peroxymonocarbonate (29,31,32). However, of particular note is the finding by Gates and coworkers that isolated PTP1B is especially reactive and reacts 7000 times faster with HCO$_4^-$ than with H$_2$O$_2$ itself (33). Although the impact of this mechanism is modulated by the equilibrium shown above, it was suggested as a possible route for PTP1B oxidation during cell signaling (20), but this proposal has not been tested experimentally. Importantly, detection of the peroxymonocarbonate species in vitro requires up to 0.1-1M concentrations, making it virtually impossible to detect directly in cellular systems. It is a highly reactive, short lived chemical species that is in equilibrium with H$_2$O$_2$, which gives the same reaction products as hydrogen peroxide. Thus, there are no specific probes or reagents that would distinguish peroxymonocarbonate.
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However, recent studies have identified links between regulation of bicarbonate levels, cell growth and cancer progression (34). Signaling through the EGF receptor, which is an important event therapeutically targeted for cancer treatment (35-37), has been directly linked to bicarbonate-regulating enzymes. Notably, carbonic anhydrase IX (CA IX), a glycoprotein with an extracellular domain that catalyzes hydration of CO$_2$ (CO$_2$ + H$_2$O $\rightarrow$ HCO$_3^-$ +H$^+$), becomes activated upon EGF ligand stimulation (38). CA IX also localizes at the leading edge of focal adhesions and its overexpression increases the rate of adhesion and spreading (39). Electroneutral sodium and bicarbonate exchanger proteins (NBCs) such as NBCn1, facilitate chloride-HCO$_3^-$ exchange with a Na$^+$/HCO$_3^-$ stoichiometry of 2:1. These membrane-bound proteins are important regulators of intracellular bicarbonate levels and help to control cellular pH (40). In MCF7 breast cancer cells, NBCn1 expression is highly upregulated upon HER2 expression (41) and is correlated with tumor progression and metastasis (42). Furthermore, genetic or pharmaceutical disruption of several NBCs (SLCA4, SLC4A7 and SLC4A9) suppresses tumor growth of breast cancer spheroids and murine xenografts (43,44) and chemical inhibition of bicarbonate influx using S0859 decreases spheroid growth of HCT116 cells (45). Considering these observations we asked whether bicarbonate can directly facilitate PTP1B oxidation in the presence of a peroxiredoxin recycling system and in a cellular setting. Surprisingly our results reveal that bicarbonate is an obligate component of cellular PTP1B oxidation and EGF responsiveness.

**Results**

**Bicarbonate potently facilitates H$_2$O$_2$-dependent inactivation of PTP1B.** Using a direct activity assay with pure recombinant PTP1B we first confirmed the previously described facilitation of H$_2$O$_2$-mediated inactivation of PTP1B by bicarbonate (33). Compared with the inefficient dose- and time-dependent direct inactivation of PTP1B by H$_2$O$_2$ (Figure 1A), inclusion of a physiological concentration of 25 mM bicarbonate potently increased inactivation (Figure 1B). Notably, bicarbonate alone had no effect (Figure 1B, control). We next examined whether inclusion of bicarbonate facilitated H$_2$O$_2$-mediated inactivation of PTP1B in the presence of a functional Trx system.

**H$_2$O$_2$ and bicarbonate in combination can overcome protection of PTP1B activity by the Trx/Prx system.** As reported previously, in the absence of bicarbonate, Prx2/Trx1/TrxR1/NADPH (at 10 µM, 2 µM, 0.5 µM and 200 µM, respectively) fully protects PTP1B against inactivation by 100 µM H$_2$O$_2$ (25). This is due to a combination of H$_2$O$_2$ removal by the peroxidase activity of Prx2 and reduction of the sulfenic acid and sulfenylamide forms of PTP1B by TrxR1 and Trx1, as illustrated in Figure 2A. In the presence of bicarbonate (25 mM), where HCO$_3^-$ can cause faster PTP1B inactivation, Prx2/Trx1/TrxR1/NADPH at the same concentrations still gave almost complete protection (Figure 2B, 1 µM TrxR1). However, by lowering the TrxR1 concentration (Figure 2B) or Trx1 concentration (Figure 2C) it was possible to obtain conditions where PTP1B was inactivated for the duration of the experiment, or inactivated at early time points, followed by a time dependent regain in activity during the course of the assay. The latter pattern is reminiscent of the transient PTP1B inactivation expected to occur in cells during an oxidative burst after growth-factor stimulation. A higher concentration of pH-adjusted bicarbonate (45 mM, pH 7.4) was also able to fully overcome the complete protection of PTP1B against 50 µM H$_2$O$_2$ provided by the cycling Prx/Trx-system, as seen by a rapid and sustained loss of PTP1B activity (Figure 2D). At intermediate concentrations of bicarbonate, maintaining the same pH, H$_2$O$_2$ caused transient PTP1B inactivation followed by recovery (Figure 2D). These results show that there is an interplay between bicarbonate, PTP1B and the Trx/Prx redox cycling systems, and that there are conditions whereby bicarbonate, presumably through the spontaneous production of peroxymonocarbonate (33), promote transient inactivation of PTP1B by H$_2$O$_2$. To rule out possible inhibitory effects of bicarbonate and H$_2$O$_2$ on the Trx system, NADPH consumption was measured using insulin as a substrate (46). The results showed no difference in consumption by bicarbonate alone or in
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**Inhibition of the bicarbonate transporter in A431 cells decreases EGF-induced phosphorylation cascades.** We next addressed whether the mechanism identified with the purified enzymes applies to cellular systems, by examining the effect of bicarbonate on protein phosphorylation cascades triggered by growth factor stimulation. We used human A431 epidermal squamous carcinoma cells, as these cells are known to inactivate PTP1B by oxidation in response to treatment with EGF (10,47). Extracellularly produced bicarbonate is transported into cells through membrane bound exchanger proteins NBCs that control intracellular bicarbonate levels and thereby cellular pH (40). We thus stimulated A431 cells with EGF ligand for 2, 4 and 6 minutes with or without 1h pretreatment with S0859, a well characterized inhibitor (S0859) of NBCs (48). As shown in Figure 3A&B, EGF ligand induced a rapid increase in total protein tyrosine phosphorylation, primarily associated with the ≈198 kDa band representing the EGF receptor (Figure 3A). This phosphorylation was notably inhibited when bicarbonate influx was blocked with S0859. The EGFR phosphorylation site Tyr 992 (pY992) previously shown to be regulated by PTP1B (49), was also significantly inhibited at all time points by S0859 (Figure 3C&D).

**Lactic acid pretreatment of A431 cells decreases EGF-induced phosphorylation.** We next investigated if protein tyrosine phosphorylation patterns were affected by physiologically relevant lactic acid concentrations, which decrease cellular bicarbonate as well as pH at events of metabolic acidosis. Lactic acid (up to 40 mM) was added to the growth medium of A431 cells 2 min prior to addition of EGF ligand. Measurements made after 5 min stimulation showed that lactic acid concentrations above 10 mM resulted in a progressive decrease in total protein tyrosine phosphorylation (Figure 4A). Time course measurements showed that addition of 15 mM lactic acid slowed the onset of EGF-dependent phosphorylation, whereas 30 mM gave very strong inhibition at all time points (Figure 4B).

**Bicarbonate depletion at constant pH decreases EGF-induced protein tyrosine phosphorylation in A431 cells.** As the results in Figures 3 and 4 could be attributed to effects of bicarbonate or to general pH effects, we next assessed whether lowering cellular levels of bicarbonate without changing the pH would still affect protein tyrosine phosphorylation patterns. A431 cells were cultured in regular DMEM medium and subsequently starved overnight in low serum bicarbonate-free HEPES-buffered (50 mM) medium, with addition of 0, 40 or 60 mM bicarbonate, adjusted to pH 7.4 and equilibrated in air with varying percentages of corresponding CO₂ levels (0, 5 and 10% respectively). The A431 cells were stimulated with EGF ligand after one day of these culture conditions and harvested 2, 4 and 6 minutes after EGF addition. Analyses of total protein phosphotyrosine revealed increases in phosphorylation at the higher bicarbonate concentration mostly pronounced for a band (not identified) at about 28 kD seen at longer exposures (Figure 5). Thus, increased cellular bicarbonate levels augment EGFR-linked protein phosphorylation in A431 cells independently of pH-derived effects.

**Oxidation of PTP1B in A431 cells during EGF stimulation depends on the presence of bicarbonate.** The observed bicarbonate-dependent increases seen in EGF-triggered phosphorylation are consistent with oxidative inactivation of PTPs. However, this is an indirect readout and other explanations are possible. We therefore looked for direct evidence of oxidative PTP1B inactivation using a cysteine-labeling assay that detects reversible thiol oxidation (50,51). The basis of the assay is to use a biotin tag that labels only reversibly oxidized thiol proteins and isolate the labeled proteins with streptavidin. Immunoblotting against the protein of interest will give a positive response only if it has been oxidized. When this procedure was applied to A431 cells maintained in regular bicarbonate-containing DMEM, we detected a time-dependent increase in oxidized PTP1B peaking at 2 minutes after EGF addition (Figure 6A). This is in agreement with earlier reports (10). Most strikingly, pretreatment with the NBC inhibitor S0859 completely abrogated the EGF-dependent PTP1B oxidation at the 2-minute time point (Figure...
Furthermore, PTP1B oxidation was not seen when the cells were treated with EGF in HEPES-buffered bicarbonate-free DMEM. Finally, addition of pH-equilibrated bicarbonate to the bicarbonate-free cells before EGF stimulation brought back oxidation of PTP1B to a level as that seen in bicarbonate-containing DMEM (Figure 6A). Thus, the presence of bicarbonate in cells is a crucial and apparently obligate component for EGF-dependent phosphorylation cascades that require oxidation and inactivation of PTP1B.

**Discussion**

Reversible oxidative inhibition of PTPs is a key regulatory event during growth factor signaling (52). The molecular mechanisms leading to PTP oxidation have remained unclear, although it is well known that RTK activation involves cellular production of \( \text{H}_2\text{O}_2 \) derived from NOX enzymes (5). Several models for PTP inactivation have been proposed, such as the “floodgate” hypothesis in which inactivation of Prxs enables oxidation of less reactive proteins (53) or redox-relay mediated oxidation via Prxs (23,24). Our results add another dimension and reveal that a concerted action of \( \text{H}_2\text{O}_2 \) together with bicarbonate is required for inactivation of PTP1B and initiation of protein phosphorylation cascades. The results with the recombinant proteins clearly showed that \( \text{H}_2\text{O}_2 \) triggered inactivation of PTP1B in the presence of a fully active and cycling Prx system only when bicarbonate was present. We used all reactants at physiologically relevant concentrations and were able to vary conditions within these to see transient inactivation and reactivation, reminiscent of a signaling step. We propose a mechanism to explain these results in which \( \text{H}_2\text{O}_2 \) can either be scavenged by Prx2 or react with bicarbonate to generate peroxymonocarbonate, which is responsible for PTP1B inactivation (Figure 2A). Increasing the bicarbonate favors the latter, whereas regeneration of reduced Prx2 by the thioredoxin system enhances \( \text{H}_2\text{O}_2 \) removal and is protective. It is possible that peroxymonocarbonate could also react with Prx2 in this system. However, we have recently found that oxidation of the reduced protein is unaffected by the presence of bicarbonate (Peskin and Winterbourn, unpublished). Although hyperoxidation was enhanced, this would cause only minor inactivation of the Prx2 under the conditions of our current study. Nevertheless, in other situations this could amplify the effect on PTP1B. Complementing these oxidative reactions are the reductive steps involving reduction of Prx2 disulfide to maintain its scavenging activity as well as recycling of PTP1B, either through a reaction of the sulfenic acid intermediate directly with TrxR1 (25) or recycling of the sulfinylamine by TrxR1 together with Trx1 or TRP14 (17). The balance between these steps determines the extent to which PTP1B is initially inactivated and subsequently reactivated as the \( \text{H}_2\text{O}_2 \) is consumed and the reducing mechanisms dominate.

To test the relevance of this mechanism of PTP1B inactivation during EGF-triggered signaling context, we modulated the cellular bicarbonate levels using three different approaches. Lowering bicarbonate uptake with the NBC inhibitor S0859 resulted in less EGFR-dependent protein tyrosine phosphorylation in A431 cells. Likewise, addition of lactic acid, which lowers bicarbonate levels by a shift in acid-base equilibrium, also decreased phosphotyrosine levels following EGF ligand stimulation in a dose dependent manner. These observations are compatible with the notion that lower intracellular levels of bicarbonate give less inhibition of cellular PTP activity, but other effects of acidification could not be excluded. However, the experiments with cells grown in pH-controlled HEPES-buffered DMEM medium revealed signaling effects of bicarbonate not related to changes in pH.

Our results collectively suggest a molecular mechanism by which physiological changes in bicarbonate levels affect growth factor signaling responses. This conclusion is substantiated by the finding that the cysteinyl-labeling assay detected EGF-dependent oxidation of PTP1B only in the presence of bicarbonate. Thus, bicarbonate seems to be an obligate factor for EGF dependent PTP1B oxidation. We hence propose a model for redox regulation of PTP1B during growth factor signaling, in which the growth factor initiates transient production of \( \text{H}_2\text{O}_2 \), which must act jointly with bicarbonate to achieve PTP1B inactivation and initiate phosphorylation cascades. For the reasons given in the Introduction, we believe that,
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although it is not possible to validate experimentally, this mechanism most likely occurs through the formation of peroxymonocarbonate (Figure 6B).

Our investigation so far has focused on PTP1B, but it is possible that the redox state of other thiol proteins could be regulated by bicarbonate. Gates and coworkers (33) showed that SHP1 is also highly reactive with peroxymonocarbonate, but this was not a general property of all the thiol proteins they studied. Thus there appears to be selectivity, and our findings give credence to the possibility that peroxymonocarbonate has a wider, yet unexplored role in H₂O₂-mediated redox signaling. They also provide a plausible explanation for how some thiol proteins that react slowly with H₂O₂ in isolation (when bicarbonate is typically not present) appear to be oxidant-sensitive during cell signaling.

Another important consideration is that intracellular bicarbonate-regulating enzymes such as CA II, IX, XII and NCBs have been shown to contribute to disease pathology, such as promotion tumor development, through a more alkaline intracellular milieu (34,38,39,54). Although most focus has been on pH effects of alkalinization of the intracellular milieu, we here propose an alternative and yet unexplored mechanism whereby redox changes associated with growth-factor responses, such as the inhibition of PTP1B, are dependent specifically upon bicarbonate.

EXPERIMENTAL PROCEDURES

Expression of recombinant proteins.

The catalytic domain (1-322) of PTP1B was subcloned into pD441 – H6 vector using PCR with primers 5’-GTAGGTCTCGTGTTGATGGAGATGGA AAGGAGTTTC-3’ and 5’-GTAGGTCTCTTA TTACCCATTGTGTGGC TCCAGG -3’ and Eco31i and Dpn1 restriction sites. The PTP1B protein was expressed and purified under reducing conditions according to previously described procedures including subsequent tag removal of (25,55). Prx2 protein was expressed and purified with a Histag which was cleaved off as previously described (56). Recombinant human Trx1 and rat TrxR1 wild type were expressed and purified as previously described (55). PTP1B was subjected to buffer exchange to remove reductant before all experiments, using Zeba Spin Desalting Columns (Thermo Scientific #87766). Protein concentrations were determined using Bradford reagents.

Treatment of PTP1B with H₂O₂ and bicarbonate. Reduced PTP1B (600 nM) was pre-incubated for 20 min in 20 mM HEPES, 100 mM NaCl buffer pH 7.4 containing 0.1 mM EDTA, 0.05% bovine serum albumin (BSA), 1 mM sodium azide, with indicated concentrations of Trx1, TrxR1, NADPH (N7505-100MG, Sigma-Aldrich) and Prx2. Sodium azide was used to inhibit any trace amounts of catalase. Each reaction mixture was exposed to H₂O₂ with and without bicarbonate for indicated times. Bicarbonate and H₂O₂ were premixed prior to treatment. Subsequent to treatment, measurement of PTP activity was performed.

PTP activity assay. PTP activity was assessed using 15 mM chromogenic substrate 4-nitrophenyl phosphate (pNPP) (P4744-1G, Sigma-Aldrich) as described previously (57). Rates of absorbance increase were measured at 410 nm at 22 °C using an Infinite M200 Pro plate reader (Tecan) and calibrated using a 4-nitrophenol standard curve.

Treatment of PTP1B with H₂O₂.

Buffer-exchanged reduced PTP1B was exposed to H₂O₂ and different components of the Trx system at indicated time points followed by addition of substrate and measurement of activity. We initially observed that incubation of PTP1B alone resulted in some time-dependent inactivation that was partially prevented in the presence of BSA, so BSA (0.05%) was added to the buffer. The activity after each H₂O₂ treatment was related to the activity of untreated PTP1B incubated for the same time. A 0.24% loss of activity was seen in control conditions at 30 min.

Insulin-coupled TrxR assay. NADPH consumption was measured through incubation of the Trx system (1 µM TrxR1, 20 µM Trx1 and 300 µM NADPH) together with 0.16 mM insulin in 20 mM HEPES, 100 mM NaCl buffer pH 7.4 containing 0.1 mM EDTA, 0.05% bovine serum albumin (BSA) with and without 25 mM bicarbonate and 100 µM H₂O₂.

Cell culture conditions and treatments. Adenocarcinoma cell line A431 cells (ATCC) were typically, unless stated otherwise, cultured in DMEM (44 mM sodium bicarbonate/5% CO₂) + 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, penicillin
and streptomycin. Cells were grown until 90% confluency and then starved for 24 h in 0.1% FCS. Pretreatment of starved cells prior to ligand stimulation was performed with indicated concentrations of either lactic acid (Sigma-Aldrich #L6402), S0859 (Sigma-Aldrich #SML0638) or sodium bicarbonate (Merck #9018415). Ligand stimulations were performed with 100 ng/ml EGF ligand (R&D Systems #236-EG-200). For cell culture conditions with varying concentrations of bicarbonate, bicarbonate-free DMEM (D5648) (0.1% (v/v) FBS, 2 mM L-glutamine, penicillin and streptomycin) was supplemented with 50 mM HEPES and 0, 40 and 60 mM bicarbonate, set to pH 7.4, and pre-equilibrated in 0, 5 and 10% CO2 respectively for 3 days prior to addition to cells, as stated in the text.

**SDS-PAGE and analysis of protein tyrosine phosphorylation.** Treated cells were washed with ice-cold PBS (pH 7.4), and lysed with lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate salt/deoxycholic acid, 150 mM NaCl, 20 mM Tris (pH 7.5), 10 mM EDTA, and 30 mM sodium pyrophosphate (pH 7.5), supplemented with 200 µM sodium ortho-vanadate and a protease inhibitors cocktail (Roche). Protein concentration of lysates was determined using the Bradford assay. Equal amounts of lysate protein were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), blocked with 5% milk in Tris-buffered saline and immunoblotted for total phosphotyrosine 4G10 (Merck #05-321) (1:1000) and EGFR pY992 (Cell Signaling). Total amount of loading was verified using Ponceau staining of blotted membranes or EGFR intensities (AF231, R&D Systems). Protein phosphotyrosine intensities were quantified using Image J and shown as a ratio of loading control, as described.

**Detection of reversible PTP oxidation in cells.** The cysteiny-labeling assay of reversibly oxidized PTP1B was performed as previously described (50,51). The lysis buffer (50 mM sodium acetate pH 5.5, 150 mM NaCl, 10% glycerol, 1% Surfact-Amp NP-40, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 50 U/ml SOD, 50 U/ml catalase, 10 mM iodoacetic acid) was degassed and placed on ice into a hypoxic glove-box station equilibrated with 100% argon. Cells cultured in 100 mm dishes were treated then transferred from a 37°C/5% CO2 environment into a hypoxic glove box station, media was carefully removed and cells were rapidly lysed with 800 µl of ice-cold lysis buffer. Lysates were transferred to amber-colored microfuge tubes and shaken for 1 h at room temperature to alkylate reduced thiols. Protein concentrations were determined and 1 mg lysate was applied to a spin column to remove excess iodoacetic acid. Tris(2-carboxyethyl)phosphine (1 mM) was then added to reduce reversibly oxidized protein thiols. Following this step, 5 mM EZ-link iodoacetyl-PEG2-biotin probe (Pierce) was added to the lysate to label the reactivated thiols. Labeled proteins were then pulled-down by streptavidin-Sepharose beads, which were washed with lysis buffer (pH 5.5), resuspended in 20 µl 4X Laemmli sample buffer, heated at 90°C for 90 s. Samples were resolved by SDS-PAGE and blotted with anti-PTP1B. Immunoblots of total cell lysates were run as controls.

**Statistical analyses.** Analyses of data were performed using GraphPad Prism with Two-way ANOVA followed by Bonferroni post hoc tests for multiple comparisons.
Acknowledgements

We thank Dr. Mark Hampton for helpful discussions.

CONFLICT OF INTEREST:
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS:
M.D. has performed all experiments, analysis of data and contributed writing the paper. Q.C. has expressed, purified, TrxR1 and Trx1. S.H.M.R has performed the CLA assay. P.E.P has expressed Prx2. B.B has performed the CLA assay and provided intellectual input. C.C.W. has designed experiments, analyzed data, provided essential intellectual input and contributed to writing the paper. E.S.JA. was overall responsible for the project and contributed to design of experiments, analyzed data, provided intellectual input and wrote the paper.

FUNDING
This work was funded by Karolinska Institutet, The Knut and Alice Wallenberg Foundations, The Swedish Cancer Society, Karolinska Institutet Research Foundations, The Swedish Research Council nr 537-2014-360, The Swedish Society of Medicine SLS-786841, Åke Wiberg Stiftelse nr M17-0101 and M18-0141, ESJA, NIH grant HL138605, American Heart Association grant 17GRNT33700265 to BB and the New Zealand Marsden Fund.

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FIGURE LEGENDS

Figure 1. Bicarbonate potentiates H$_2$O$_2$-dependent inactivation of PTP1B. A: Recombinant PTP1B (600 nM) was treated with increasing concentrations of H$_2$O$_2$, 0 µM buffer control (●), 3,4 µM (○), 6,3 µM (●), 12,5 µM (▼) 25 µM (▲), 50 µM (■) (20 mM HEPES, 100 mM NaCl buffer pH 7.4 containing 0.1 mM DTPA, 0.05% bovine serum albumin (BSA), 1 mM sodium azide) and then assayed for PTP activity at indicated times. PTP1B activity is given in min$^{-1}$ (mol product/mol enzyme/min). Data points represent means ± SD (n=3). B: PTP1B was treated as in A but in the presence of 25 mM bicarbonate (final concentration after adding H$_2$O$_2$/bicarbonate and assay buffer). Data points represent means ± SD (n=4). A representative run is presented in supplementary Fig. S1A and B.

Figure 2. Modulation of bicarbonate- and H$_2$O$_2$-dependent inactivation of PTP1B in the presence of a Prx2/TrxR1/Trx1 redox system. A: Scheme showing relevant reactions in the experimental PTP1B/Prx2/thioredoxin system. S$_{ox}$ of PTP1B could be either the sulfenic acid or sulfenylamide species, with the former also reducible by TrxR/NADPH in the absence of Trx. B: PTP1B (600 nM) was treated with 50 µM H$_2$O$_2$ together with 25 mM bicarbonate in the presence of Trx1 (5 µM), Prx2 (10 µM), NADPH (300 µM) and increasing TrxR1 concentrations of 0 nM (■), 2 nM (▲), 10 nM (▼), 50 nM (●), 250 nM (○), 1 µM (□) and with 1 µM TrxR1 using buffer-treated control without H$_2$O$_2$ (●). PTP activity was measured after indicated times. Data points represent means ± SD (n=3). C: Analyses were performed as in (B) with TrxR1 (1 µM) and increasing Trx1 concentration, 0 µM (●), 0,5 µM (▼), 1 µM (▲), 2 µM (■) and 5 µM (●) (n=1). D: Analyses performed as in (A) with TrxR1 (15 nM) and increasing concentrations of bicarbonate as indicated, 0 mM (■), 5 mM (▲), 10 mM (▼), 15 mM (●), 25 mM (○), 35 mM (□), 45 mM (△) (n=1).

Figure 3. Pharmacological inhibition of bicarbonate cotransporters in A431 cells decreases EGF ligand-induced phosphotyrosine formation. Overnight starved A431 cells in regular bicarbonate-containing DMEM 0.1% FCS) were pretreated with the NBC inhibitor S0859 (100 µM) or DMSO control for 1h prior to EGF stimulation for 2, 4 and 6 min. Cell extracts were separated by SDS-PAGE and western blotted for (A) total phosphotyrosine, EGF receptor and (C) specific phosphorylation of the EGF receptor Tyr 992 (pY992) residue. Bar diagrams show densitometry ratios for total signal over all phosphotyrosine bands (B), or the specific pY992 signal (D), relative to the immunoblot staining for the EGF receptor. Every ratio is compared to that in unstimulated control which was set to 1. Results are mean ± SD for 3 independent experiments with symbols showing individual results. Statistically significant differences are indicated (*=P < 0.05 ). The membranes stained with Ponceau for total protein loading are shown in supplementary Fig. S3.

Figure 4. Treatment of A431 cells with lactic acid prior to EGF ligand stimulation results in less phosphorylation. A: Concentration dependence. Serum-starved A431 cells in regular DMEM containing bicarbonate were pretreated for 2 min with 0, 5, 10, 20 and 40 mM lactic acid and subsequently stimulated with EGF (100 ng/ml) for 5 min. Lysates were analyzed for total phosphotyrosine as in Figure. B: Time course. A431 cells were pretreated for 2 min with 0, 15 and 30 mM lactic acid and subsequently stimulated with EGF (100ng/ml) for 2, 4 and 6 minutes. Lysates were analyzed for total phosphotyrosine. Right hand panels in (A) and (B) show densitometry analyses as in Fig 3 with symbols showing individual results (n=3, ± SD; *P < 0.05). The membranes stained with Ponceau for total protein loading are shown in supplementary Fig. S4.
Figure 5. **Bicarbonate increases total phosphorylation in A431 cells after EGF ligand stimulation.**
A: A431 cells were grown and incubated overnight in low serum HEPES-buffered (50 mM) DMEM at pH 7.4 with 0, 40 and 60 mM bicarbonate added and equilibrated with 0, 5 and 10% CO₂ respectively, as indicated. The cells were then stimulated with EGF and analyzed for total phosphotyrosine. B: Densitometry for 3 independent experiments, of short and long exposed membranes, for high molecular weight (HMW) phosphorylated proteins and ~28 kDa band, respectively (dashed rectangles in A) quantified in relation to Ponceau staining with 2, 4 and 6 minute time points combined (n=3, ±SD *P < 0.05). Symbols indicate individual results.

Figure 6. **A: Bicarbonate is required for EGF dependent reversible PTP1B oxidation in A431 cells.** A431 cells were incubated overnight either in low serum regular DMEM containing bicarbonate or in HEPES-buffered DMEM (50 mM pH 7.4). Pretreatment of the cells in bicarbonate-containing DMEM with the NBC inhibitor S0859 (50 µM) was for 1h prior to stimulation. Bicarbonate (60 mM) was added to cells in HEPES-buffered DMEM and subsequently stimulated. At indicated times after EGF stimulation, cells were subjected to the cysteinyllabeling assay using biotinylated iodoacetyl-PEG2-biotin for analysis of reversible PTP1B oxidation. Biotinylated proteins were purified on streptavidin-Sepharose beads, resolved by SDS/PAGE and visualized using antibodies against PTP1B. PTP1B control levels were determined from total cell lysate by SDS/PAGE and blotting against PTP1B. A representative western blot of three independent experiments is shown. **B: Model for regulation of PTP1B activity during growth factor signaling.** EGFR activation induces a transient burst of H₂O₂, which reacts with bicarbonate to give PTP1B oxidation via peroxymonocarbonate (red) and activation of phosphorylation pathways (green arrow). Prxs compete for the H₂O₂ and the Trx-system decreases the availability of H₂O₂ by supporting the Prx cycle and also acts by reactivating oxidized PTP1B. See text for further details.
PTP1B oxidation and signaling by EGF requires bicarbonate
Figure 2

A) HCO₃⁻ + H₂O₂ → HCO₃⁻ + H₂O

B) PTP1B activity (min⁻¹)

C) PTP1B activity (min⁻¹)

D) PTP1B activity (min⁻¹)
Figure 3

A) EGF 0 2 4 6 0 2 4 6 min
IB:Total pTyr
kDa 62 49 38 28
IB:EGFR kDa 198

B) Total pTyr/EGFR

C) IB:pTyr992

D) pTyr992/EGFR

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Figure 4

A) mM Lactic acid

| EGF | 0 | 5 | 10 | 20 | 40 |
|-----|---|---|----|----|----|
| IB: Total pTyr | 198 | 198 | --- | --- | --- |
| IB: EGFR | 198 | 198 | --- | --- | --- |

B) mM Lactic acid

| EGF | 0 | 15 | 30 |
|-----|---|----|----|
| IB: Total pTyr | 198 | 198 | 198 |
| IB: EGFR | 198 | 198 | 198 |

Graph:

- X-axis: mM Lactic acid
- Y-axis: Total pTyr/EGFR

- 0mM Lactic acid
- 15mM Lactic acid
- 30mM Lactic acid
Figure 5

A) Table showing the % CO₂ and EGF (min) for different HCO₃⁻ concentrations:

| HCO₃⁻ mM | 0 | 40 | 60 |
|----------|---|----|----|
| % CO₂   | 0 | 5  | 10 |
| EGF (min)| 0 | 2  | 4  |
| IB:Total pTyr | | | |
| Short exp. | 49 | | |
| IB:EGFR | 198 | | |

B) Ponceau staining and pTyr/ponceau graph:

- HCO₃⁻ % CO₂ (mM):
  - 0, 0%
  - 40, 5%
  - 60, 10%

- Graph showing pTyr/ponceau values for Short exp., Long exp., and I-20 BA Band.
Figure 6

A) EGF oxidation and signaling by EGF requires bicarbonate

B) EGF-mediated bicarbonate production and signaling in PTP1B-mediated proliferation and migration.
Bicarbonate is essential for protein tyrosine phosphatase 1B (PTP1B) oxidation and cellular signaling through EGF-triggered phosphorylation cascades
Markus Dagnell, Qing Cheng, Syed Husain Mustafa Rizvi, Paul E. Pace, Benoît Boivin, Christine C. Winterbourn and Elias S.J. Arnér

J. Biol. Chem. published online June 13, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.009001

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