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Oxidation inhibits PTH receptor signaling and trafficking

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Abstract

Reactive Oxygen Species (ROS) increase during aging, potentially affecting many tissues including brain, heart, and bone. ROS alter signaling pathways and constitute potential therapeutic targets to limit oxidative damaging effects in aging-associated diseases. Parathyroid hormone receptors (PTHR) are widely expressed and PTH is the only anabolic therapy for osteoporosis. The effects of oxidative stress on PTHR signaling and trafficking have not been elucidated. Here, we used Fluorescence Resonance Energy Transfer (FRET)-based cAMP, ERK, and calcium fluorescent biosensors to analyze the effects of ROS on PTHR signaling and trafficking by live-cell imaging. PTHR internalization and recycling were measured in HEK-293 cells stably transfected with HA-PTHR. PTH increased cAMP production, ERK phosphorylation, and elevated intracellular calcium. Pre-incubation with H_2O_2 reduced all PTH-dependent signaling pathways. These inhibitory effects were not a result of PTH oxidation since PTH incubated with H_2O_2 triggered similar responses. PTH promoted internalization and recycling of the PTHR. Both events were significantly reduced by H_2O_2 pre-incubation. These findings highlight the role of oxidation on PTHR signaling and trafficking, and suggest the relevance of ROS as a putative target in diseases associated with oxidative stress such as age-related osteoporosis.

Keywords

PTH; PTH receptor; hydrogen peroxide (PubChem CID:784); oxidative stress; signaling; trafficking

1. Introduction

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), are generated during mitochondrial oxidative metabolism and also produced by exogenous sources. Oxidative stress arises from an imbalance between an increase of ROS and the antioxidant cellular defensive mechanisms [1,2]. Oxidative stress causes nucleic acid, lipid and protein damage [2], and is involved in the pathogenesis of several diseases, including age-related bone loss in osteoporosis and some types of nephropathies [3–5]. During skeletal aging, accumulation of ROS affects several regulatory processes such as osteoblast and osteoclast apoptosis,

osteoblastogenesis, and adipogenesis, involved in bone homeostasis. Disruption of these activities adversely affects bone mass and strength [6,7]. In addition, ROS play an essential role in the pathology of several renal diseases including ischemic acute renal failure, renal graft rejection, acute glomerulonephritis, toxic renal diseases [8], and diabetic nephropathy [5]. ROS-dependent oxidation of proteins results in structural modifications that may change their function. These alterations partially result from changes in signaling pathways that are increased or decreased by ROS. Identifying these pathways and targets represent potential therapeutic opportunities to limit the consequences of oxidative damage in ROS-associated diseases [1].

Parathyroid hormone (PTH) receptor type I (PTHR) is a seven transmembrane G-coupled protein receptor (GPCR) that is highly expressed in the kidney and bone, where it is a key component of bone development and mineral-ion balance, and is a target for current and proposed drugs to treat bone and mineral disorders [9]. The PTHR is a member of Family B GPCRs and signals through Gs and Gq, which in turn activate adenylyl cyclases/cAMP/PKA and phospholipase C/inositol phosphates (IP_3)/calcium/PKC cascades, respectively [10]. ERK1/2 MAP kinase is another signaling pathway triggered by activated PTHR that regulates bone and renal functions [11,12]. The ligands for PTHR, PTH and PTH-related protein (PTHrP), are involved in the etiology of processes such as osteoporosis and hypercalcemia of malignancy. PTH(1-34) (teriparatide) is employed therapeutically for management of osteoporosis and PTHrP(1-34) (abaloparatide) is presently in clinical studies. After ligand binding and activation, most GPCRs are phosphorylated, bind β-arrestins, and are endocytosed. Desensitized receptors are subsequently recycled to the plasma membrane or trafficked to lysosomes for degradation [13].

Based on the pathogenic potential of ROS and given the essential role of PTHR signaling in bone and renal physiology we analyzed the effects of oxidative stress as found in osteoporosis and diabetic nephropathy on PTHR activity. To accomplish this, we characterized the actions of ROS, modeled with H_2O_2, on PTHR signaling and trafficking. Here, we show that PTH-dependent cAMP, ERK1/2, and calcium signaling, as well as PTHR endocytosis and recycling, are inhibited by H_2O_2.

2. Materials and Methods

2.1 Cell culture and transfection

HEK-293 cells were stably transfected with human influenza hemagglutinin (HA)-PTHR (HEK-293R) [14] and cultured in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.75 mg/ml geneticin. HEK-293R cells express 6.0×10^5 PTHR/cell, with an average Kd of 14.2 nM. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. Cells were pre-incubated for 15 min with different concentrations of H_2O_2 (1–500 μM) followed by stimulation with 10 nM–1 μM [$Nle^{8,18}$,Tyr^{34}]-PTH(1-34) (hereafter NNT-PTH) (Bachem, Torrance, CA), an oxidant-resistant PTH peptide.

For transient transfections, HEK-293R cells were grown on poly-*D*-lysine-coated glass 25-mm coverslips for 12 h prior to transfection with FuGENE 6 (Roche Applied Science) in

complete medium. After 24 h, coverslips in HEPES/ bovine serum albumin buffer (pH=7.4) (HEPES buffer containing 0.1% (w/v) BSA) were transferred to an Attofluor chamber (Invitrogen, Carlsbad, CA) for live-cell imaging.

2.2 Fluorescence Resonance Energy Transfer (FRET)

HEK-293R cells were transiently transfected with the cAMP biosensor EPAC [15] or with the ERK-NES biosensor [16]. cAMP formation and ERK activation were measured in real time in live cells by fluorescence resonance energy transfer (FRET) as described [17]. Cells were observed using a 40× 1.30 NA oil immersion objective on a Nikon A1s confocal microscope attached to a Ti-E inverted base. FRET signals are reported as the normalized FRET ratio (nFRET) of the yellow fluorescent protein (YFP) and cyan fluorescent protein emission ratio (F_{YFP}/F_{CFP}).

2.3 Intracellular calcium

Intracellular calcium activity ($[Ca^{2+}]_i$) was measured with Fluo-4 AM (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Briefly, HEK-293R cells were cultured on poly-*D*-lysine-coated glass 25-mm coverslips with 2 μM Fluo-4 AM in Hanks' balanced salt solution (HBSS) (Invitrogen) at 22 °C for 45 min. Cells were washed three times with HBSS and further incubated at 22 °C for 30 min. Calcium measurements were performed with a Nikon A1s inverted fluorescence microscope. Fluorescence was recorded at 1-s intervals for up to 20 min. At least 30–40 cells were counted under each condition. Intracellular calcium activity was calculated from the following equation: $[Ca^{2+}]_i = Kd \times (F - Fmin)/(Fmax - F)$, where F is the measured fluorescence intensity; Fmax is the fluorescence measured after addition of 10 μM ionomycin (maximum $[Ca^{2+}]i$ increase); Fmin is the fluorescence measured after addition of 10 mM EGTA (minimum $[Ca^{2+}]_i$), and Kd is the dissociation constant of the dye-Ca^{2+} complex (520 nm) [17].

2.4 Receptor internalization

PTHR internalization was measured in HEK-293R cells seeded on poly-*D*-lysine-coated 24-well plates. Confluent cells were treated with NNT-PTH in the presence or absence of H_2O_2 and subsequently fixed with 3.7% paraformaldehyde at room temperature. After 3 washes with phosphate-buffered saline (PBS) [pH=7.4] cells were blocked with 1% BSA for 45 min and incubated with a rabbit polyclonal anti-HA antibody (Covance, Berkeley, CA; 1:1000) for 1 h at room temperature.

Cells were then washed with PBS, re-blocked with 1% BSA for 15 min, and incubated with an anti-rabbit IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000). After washing, alkaline phosphatase substrate *p*-nitrophenyl phosphate was added for 30 min, 100 μL of the reaction mixture were transferred to a 96-well plate, and absorbance was measured at 405 nm [17].

2.5 Statistical analysis

Data are presented as the mean ± SEM from 3 independent experiments. Multiple comparisons were evaluated by nonparametric Kruskal-Wallis with *post-hoc* analysis using

Dunn's multiple comparison test (Prism, GraphPad). Differences greater than $P \leq 0.05$ were assumed to be significant.

3. Results

3.1 PTH-dependent signaling is inhibited by H_2O_2

NNT-PTH promptly augmented intracellular cAMP as reflected by the rapid induction of EPAC (Fig. 1A) and ERK-NES (Fig. 1B) and augmented intracellular Ca^{2+} (Fig. 2A, 2B).

To define the effects of oxidative stress on PTHR signaling, we analyzed the response of exposure to 100 μM H_2O_2, which we previously demonstrated to be a maximally effective dose on ERK1/2, p38, JNK, p66Shc, and Akt kinase phosphorylation [18]. Pre-incubation of HEK-293R cells with 100 μM H_2O_2 significantly decreased NNT-PTH-dependent cAMP intracellular accumulation and ERK phosphorylation (Fig. 1A and B). Similarly, H_2O_2 dampened the magnitude of NNT-PTH stimulation cytoplasmic calcium activity (Fig. 2).

Oxidation of PTH results in loss of biological activity [19–22]. To determine whether the reduced PTHR signaling following H_2O_2 treatment was caused by PTH oxidation, we pre-incubated the NNT-PTH peptide with H_2O_2. No apparent differences of cAMP accumulation were observed after exposure of H_2O_2-pre-incubated NNT-PTH compared to NNT-PTH controls (HBSS) (Fig. 3).

3.2 H_2O_2 modifies PTH receptor trafficking

We next evaluated the effects of H_2O_2 on PTHR trafficking by measuring internalization and recycling to the plasma membrane. Under control conditions up to 30% of PTHR internalized upon NNT-PTH stimulation. Receptor endocytosis was greater at 100 nM and 1 μM compared to 10 nM NNT-PTH. In contrast, H_2O_2 pre-incubation of HEK-293 cells decreased receptor internalization in a concentration-dependent manner (Fig. 4A). At 10 nM NNT-PTH, H_2O_2 virtually abolished PTHR sequestration, while at 100 nM or 1 μM inhibition was 35–40%.

Once internalized, resensitized receptors recycle to the plasma membrane, allowing cells to reuse receptors efficiently [13]. Under control conditions, *i.e.*, in the absence of H_2O_2, PTHR recycling to the plasma membrane was essentially complete within 30 min after peptide removal. In striking contrast, pre-incubation with H_2O_2 abolished PTHR recycling (Fig. 4B).

4. Discussion

PTHR is expressed at high levels in bone and kidney and at lower levels in a number of tissues, including blood vessels, heart, liver, and nervous system mediating a large array of endocrine or paracrine/autocrine functions in response to systemic PTH or locally produced PTHrP, respectively [23]. Many diseases associated with oxidative stress affect organs where PTHR acts and, despite the key actions of this receptor in these organs, the effects of oxidative stress on PTHR signaling have not been studied. Here, we show that H_2O_2

decreases PTH-dependent PTHR signaling and trafficking, suggesting a new target for treatment in oxidative stress-mediated diseases involving PTHR signaling.

Patients with advanced stages of renal disease, involutional osteoporosis, cardiovascular diseases, among others, exhibit increased levels of systemic oxidative stress with the potential to oxidize hormones such as PTH. Several reports show that oxidized PTH has weakened biological activity as a consequence of diminished PTHR binding [20,24,25]. PTH has two methionine residues at positions 8 and 18 and studies by independent groups have shown that oxidation of PTH diminishes its interaction with PTHR, does not stimulate the PTH to generate cAMP, and triggers abnormal calcium, phosphorus and vitamin D biological responses [25–27]. The secondary structure of PTH is essential for its receptor binding, with methionine-8 critical for the folding and biological activity of the hormone [19,25]. Thus, oxidation of methionine-8 is implicated both in binding and in activation of adenylyl cyclase [25]. To avoid the loss of biological activity due to PTH oxidation, here we used a modified peptide, where methionines at positions 8 and 18 are replaced by norleucine [$Nle^{8,18}$,Tyr^{34}]-PTH(1-34) [28]. In addition, comparable cAMP responses obtained in experiments with or without H_2O_2 preincubation with NNT-PTH suggest that decreased PTHR signaling and trafficking following treatment with H_2O_2 are caused by oxidation of the receptor or downstream effectors and not to ligand oxidation.

PTH binding to PTHR stabilizes the receptor in an active conformation that leads to interaction of the intracellular loop regions of PTHR with Gs and Gq proteins, which results in the initiation of intracellular signaling [10]. Cysteines are thought to play a critical role in receptor function and conformation by modifying structure through the introduction of disulfide bonds or by acting as reactive enzyme centers, together with amino acids like histidine and asparagine [29,30]. The high predisposition of ROS to affect cysteine residues is especially relevant given the role of GPCR and G-protein cysteine residues in the formation of intra- and inter-molecular disulfide bridges and receptor complexes, formation of ligand binding domains, and stabilization of protein conformations through modifications such as palmitoylation and prenylation, which facilitate downstream signal transduction [31]. PTHR harbors eight extracellular that have been shown to be essential for receptor function [32], and five additional intracellular cysteines distributed in loops 1 and 3, transmembrane domain 7, and the C-terminal intracellular tail.Cystein-217 in intracellular loop 1 is necessary for adenylyl cyclase/cAMP signaling [33]. It is possible though, that oxidation of cysteines in PTHR changes conformation of the receptor or modifies binding domains resulting in reduced interactions with signaling partners. Supporting this notion, signaling from members of the tyrosine kinase receptor family such as the epidermal growth factor receptor (EGFR), has shown to be regulated by cysteine oxidation [34], and oxidative stress generated by H_2O_2 results in an aberrant pattern of receptor phosphorylation [35]. In these studies H_2O_2 functions as a secondary messenger to regulate intracellular signaling cascades, largely through the modification of specific cysteine residues such as Cys-797 in the EGFR active site within redox-sensitive protein targets [36]. Exogenous ROS also cause direct cysteine oxidation in GPCRs, including the β2 adrenergic receptor [37], in which cysteine residues are key regulators of adenylyl cyclase stimulation [38]. GPCR signaling can also be modified by ROS targeting of receptor protein partners. Oxidative stress induces G protein-coupled receptor kinases-2 (GRK2) translocation to the membrane, where GRK2

interacts with Gq/11α, uncoupling it from the dopamine D1 receptor and leading to reduced phospholipase C signaling [39]. Similar mechanisms to those described above may explain the decreased PTHR signaling in following H_2O_2 exposure.

Our data not only show decreased PTHR signaling but also diminished receptor internalization and recycling. In contrast to most receptors studied to date, in which signaling is rapidly extinguished by receptor endocytosis, PTHR signaling in response to PTH continues even after internalization of the activated receptor [40]. Prolonged PTHR intracellular signaling involves internalization and stabilization of an active [PTH-PTHR] complex in early endosomes [40,41]. Thus, inhibition of PTHR internalization by H_2O_2 as shown here may result in moderated signaling due to reduced formation of the endocytic PTHR complex. In this regard, several reports describe inhibition of endocytosis of a variety of molecules, including receptors, by pretreating cells with H_2O_2 [42–44]. Furthermore, PTHR endocytosis occurs in a clathrin-dependent manner [45] and clathrin-dependent endocytosis is disrupted under oxidative stress by a mechanism involving tyrosine phosphorylation of clathrin heavy chain [46]. Once the PTHR internalizes into early endosomes, it subsequently traffics to an actin-sorting nexin 27-retromer tubule complex (ASRT), a sorting platform on early endosomes that promotes recycling of surface receptors and has recently been described to mediate rapid PTHR recycling to the surface [41,47,48]. Interestingly, the retromer complex has been described as a target of oxidative stress. Oxidative stress switches the glucose transporter 4 (GLUT4) sorting direction to lysosomes through inhibition of retromer function, by a protein kinase CK2-dependent mechanism, instead of promoting translocation from intracellular compartments to the plasma membrane [49]. In this respect, oxidative stress may contribute to the altered receptor trafficking and the observed impaired cellular signaling.

Although further investigation will be required to elucidate the precise molecular mechanisms that mediate decreased PTHR signaling and internalization and recycling, the present study reveals novel responses to oxidative stress by the PTHR and suggests a novel mechanism of pathological PTHR dysfunction in oxidative stress-mediated diseases.

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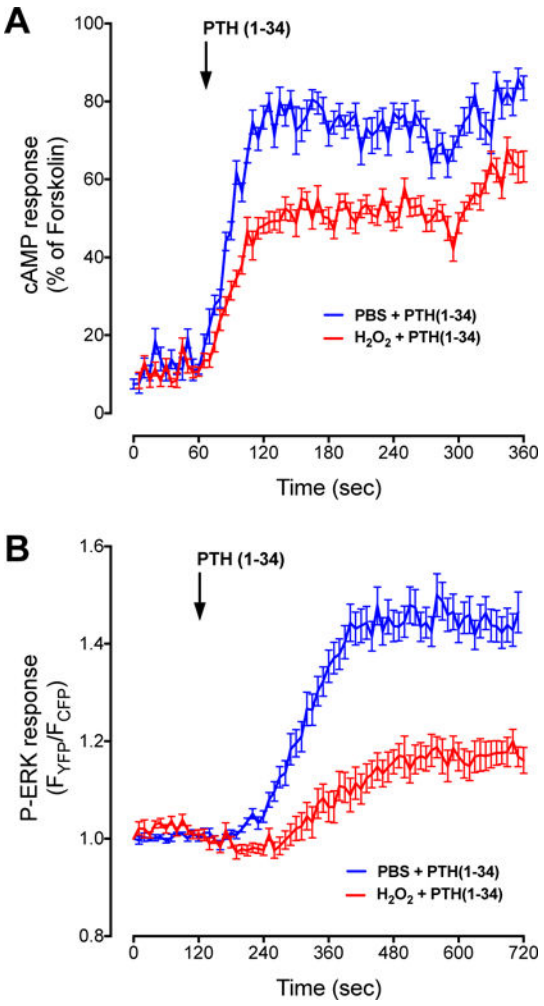

Fig. 1. H_2O_2 suppresses PTH-stimulated cAMP formation and ERK activation

A) HEK-293R cells were transiently transfected with an EPAC FRET sensor to measure cAMP. After a 60-sec baseline recording 100 nM NNT-PTH in PBS was added and recording continued for an additional 5 min. Pretreatment with 100 μM H_2O_2 for 15 min decreased cAMP formation. Data are the mean ± SEM of N = 3 independent experiments with n = 20 (PTH[1-34] or 30 (H_2O_2 + PTH[1-34]). **B)** pERK1/2 activation was similarly measured using the ERK-NES FRET sensor. Here, a 2-min baseline recording was followed by introduction of 100 nM NNT-PTH. 100 μM H_2O_2 for 15 min decreased pERK1/2. Data are the mean ± SEM of N = 3 independent experiments with n = 22 (PTH[1-34] or 15 (H_2O_2 + PTH[1-34]).

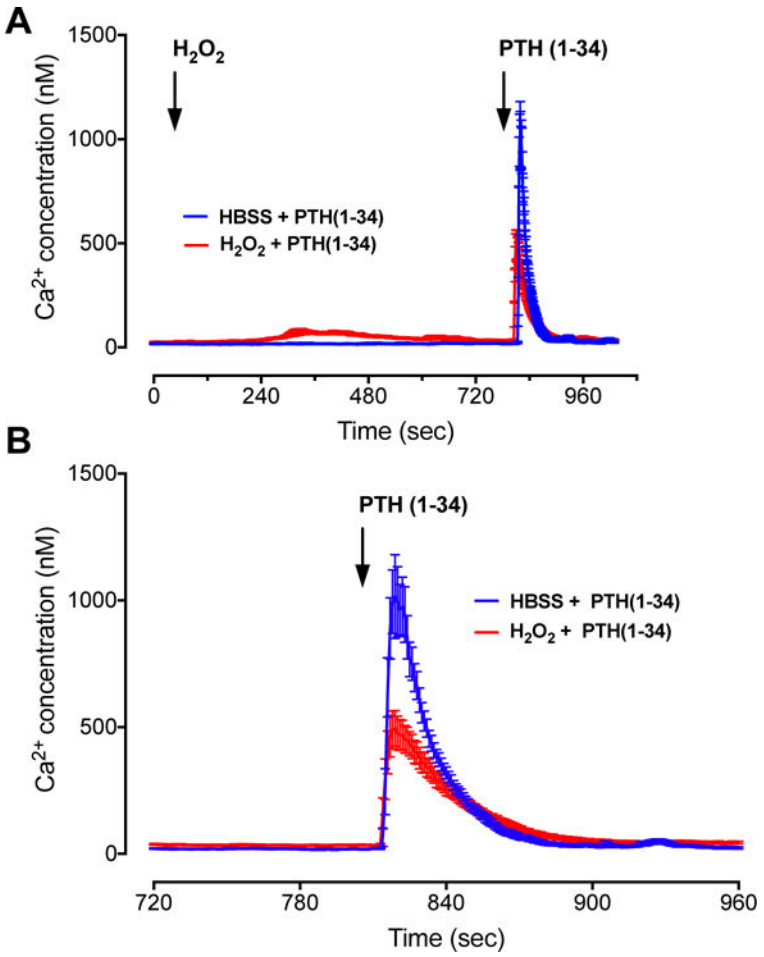

Fig. 2. Intracellular calcium accumulation upon PTH stimulation is inhibited by preincubating cells with H_2O_2

A) 100 nM NNT-PTH caused rapid increases in Fluo-4 AM fluorescence in HEK-293R cells, corresponding to an elevation of intracellular calcium activity. Pretreatment with 100 μM H_2O_2 for 15 min decreased calcium signaling triggered by NNT-PTH. **B)** Detailed changes of Fluo-4 AM after PTH stimulation. Data represent the mean ± SEM of N = 3 independent experiments; n = 17 for H_2O_2 + NNT-PTH and n = 20 for HBSS + NNT-PTH.

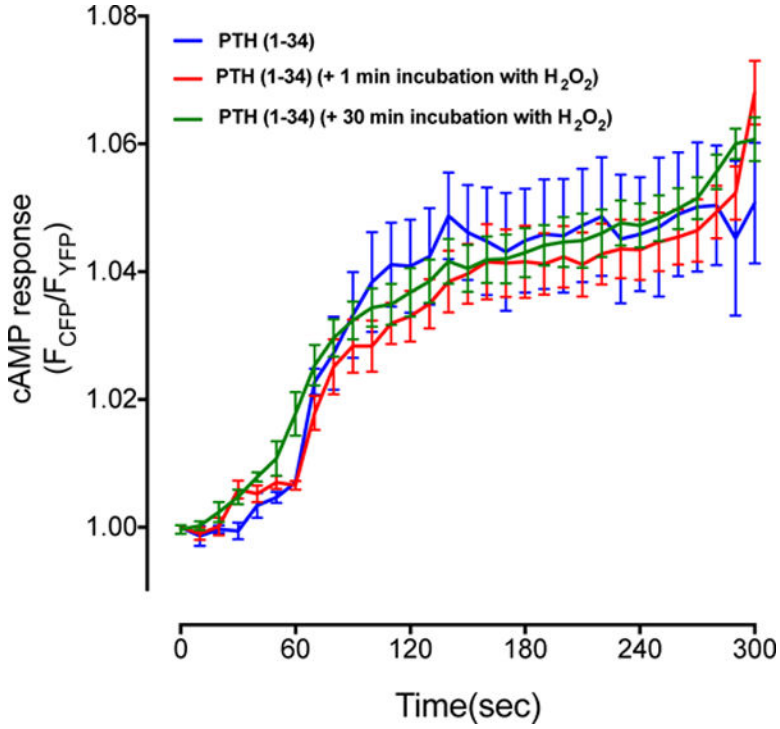

Fig. 3. Ligand-dependent accumulation of cAMP is not inhibited by incubation of PTH with H_2O_2

100 nM NNT-PTH increased cAMP accumulation in HEK-293R cells. No significant changes in cAMP responses were observed after preincubating NNT-PTH with 100 μM H_2O_2 for 5 or 30 min. Data represent the mean ± SEM of N = 3 independent experiments n = 10 cells for each condition.

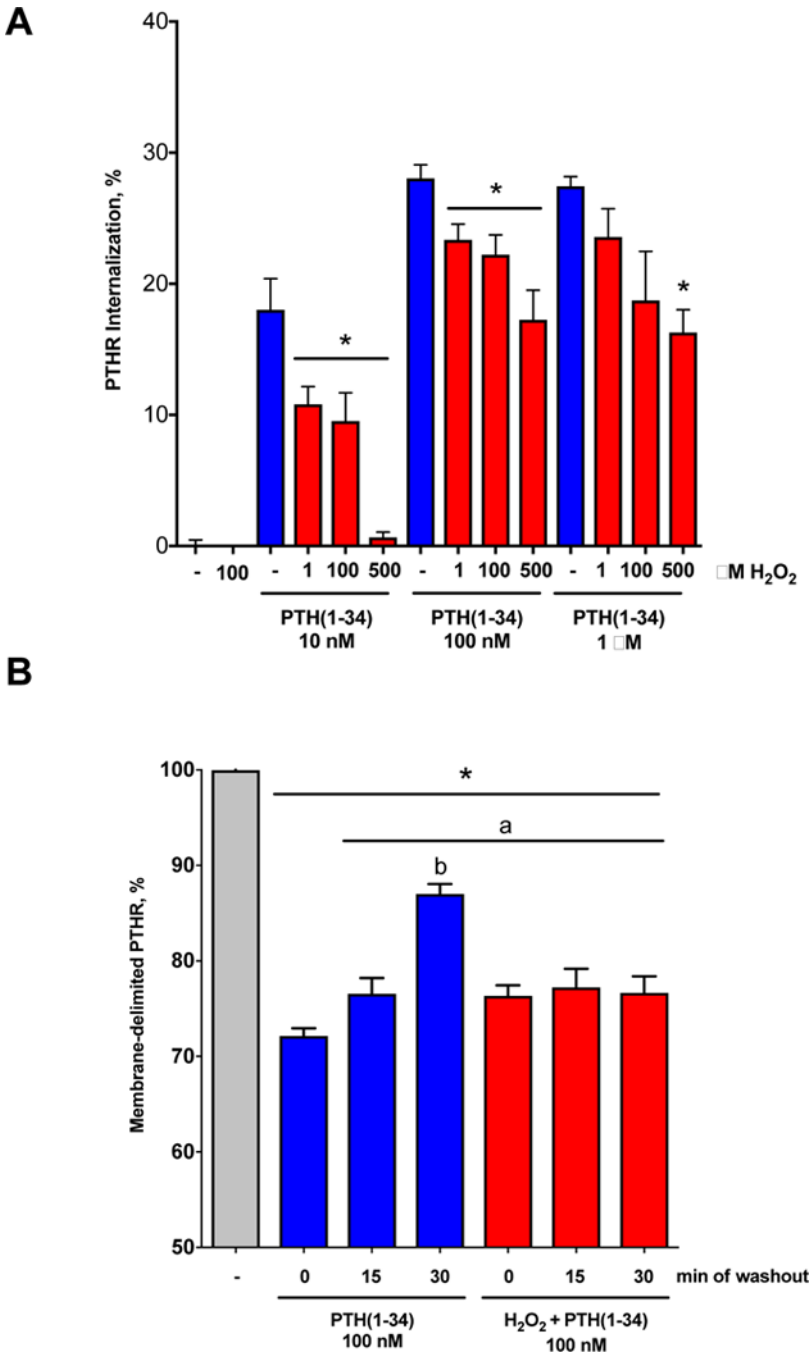

Fig. 4. PTHR internalization and recycling upon PTH stimulation are inhibited by H_2O_2

A) NNT-PTH at 10 nM, 100 nM or 1 μM concentrations induced PTHR internalization within 30 min. Pretreating cells with 1–500 μM H_2O_2 significantly inhibited PTHR endocytosis. **B)** PTHR internalized by 100 nM NNT-PTH recycles to the cell membrane in a time-dependent manner following peptide washout. PTHR recycling was annihilated by pretreating cells for 15 min with 100 μM H_2O_2. Data represent the mean ± SEM of N = 3

independent experiments. *P <0.05 *vs.* no PTH (−); [a]P <0.05 *vs.* NNT-PTH (0); [b]P <0.05 *vs.* corresponding H_2O_2.