学 位 申 請 論 文

Co-expression of histamine H3 receptor (H_3R) decreases uptake activity and membrane expression of norepinephrine transporter (NET)

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ヒスタミン H3 受容体の共発現はノルエピネフリントランスポーターの取り込み活性と膜発現を減少させる

文 学方

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Introduction

Norepinephrine (NE) is a major chemical messenger in central nervous system and sympathetic synapses and mediates many important physiological functions including arousal, cognition, emotion, feeding behavior, blood pressure and heart rate [1-3]. Dysregulation of NE system has been implicated to contribute to the pathogenesis of several disorders, such as depression, attention deficit hyperactivity disorder (ADHD), schizophrenia and orthostatic intolerance (OI) [1, 4, 5]. However, the precise mechanism responsible for regulating synaptic NE levels has not yet been elucidated in detail.

Norepinephrine transporter (NET), a presynaptic membrane protein, belongs to solute carrier family 6, which also includes transporters for dopamine, serotonin, GABA and glycine. By facilitating the reuptake of released NE back into presynaptic terminal, NET contributes to the regulation of noradrenergic tone [6]. Targeted deletion of the NET gene in mice was previously shown to induce profound alterations in NE homeostasis and mimicked the effect of antidepressant [7]. Genetic variants of NET that affect membrane expression and function have been linked to OI and other diseases [8-10]. NET is also an important target for antidepressants and stimulants [11], which increase extracellular NE levels by exerting their actions through NET to either inhibit NE uptake or increase the release of NE from presynaptic terminals [12]. The importance of NE uptake in NE homeostasis underscores the need for understanding processes that regulate NET.

Previous studies demonstrated that NET can be regulated through different manners. Some receptors, including those for acetylcholine [13], insulin [14] and angiotensin II [15] have been implicated in the regulation of NET. Moreover, the existence of physical complexes containing NET suggests that NET function can also be modulated by the interaction with different protein molecules, such as PICK1, PP2A, Hic-5, syntaxin1A, α-synuclein and neurokinin-1 receptor [16, 17].

Histamine H3 receptor (H_3R) is one of the four histamine receptors and is recognized to be a predominantly presynaptic autoreceptor that regulates the release of histamine [18]. On the other hand, H_3R has also been detected on nonhistaminergic neurons which influences the release of other neurotransmitters, including NE, acetylcholine, dopamine, serotonin, GABA, and glutamate[19-25]. A previous study reported that NE release was inhibited by the activation of H_3R [20], and the release of NE from the ischemic heart was augmented in mice lacking H_3R [26]. However, to control the homeostasis of NE in the synaptic cleft, whether NET function and expression can be regulated by co-expression of H_3R is still not clear. To investigate the effects of co-expression of H_3R on NET function, we utilized Chinese hamster ovary (CHO) cells stably expressing NET and showed that NET function and expression can be modulated by H_3R when co-expressed.

Materials and Methods

Cell culture and generation of Chinese hamster ovary (CHO) cell stably expressing rat NET

CHO cells were maintained in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 μg/ml streptomycin in humidified incubator with 5% CO_2 at 37℃. For preparation of cell line that stably expressed rat norepinephrine transporter (rNET), CHO cells at subconfluence were transfected with cDNA of rNET using FuGENE6 transfection reagent (Promega Corporation, Madison, WI, USA) according to the manufacturer's directions. Thereafter, cells were diluted sequentially and seeded on 96-well plates with α-MEM containing G418 (Promega Corporation, Madison, USA) to select transfected cell lines. The cell line showing uptake of [^{3}H] NE (1.18 TBq/mmol, GE healthcare Bioscience, Inc., Buckinghamshire, UK) was designated as NET/CHO [27].

Uptake assay

NET/CHO cells were transfected with either pcDNA3 vector (Invitrogen, Life Technology, Carlsbad, CA, USA), rat H_3R (rH_3R coding region inserted in pcDNA3) or rat H_3R-antisense (rH_3R antisense inserted in pcDNA3). Forty-eight hours after transfection, the cells were washed with Krebs Ringer HEPES-buffered (KRH; 125 mM NaCl, 5.2 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5mM glucose, and 20 mM HEPES, pH 7.3) solution, followed by incubating for 10 min at 37 □ with 10 nM [^{3}H] NE in KRH containing 0.1 mM sodium ascorbate (Katayama

Chemical industries Co., Osaka, Japan), 0.05 mM pargyline hydrochloride (Nacalai Tesque, Inc., Kyoto, Japan) and 10 mM OR486 (Tocris Cookson Ltd., Avonmouth, UK). After this incubation, reaction was rapidly terminated by rinsing 3 times with ice cold KRH buffer. Cells were then solubilized in 1 M NaOH and neutralized with the same amount of 1 M HCl. The radioactivity in the solubilized cells was measured by liquid scintillation counter. Nonspecific uptake was determined in the presence of 100 nM cocaine (Takeda Chemical Industries Ltd., Osaka, Japan) and NET mediated specific uptake was defined by subtrating nonspecific uptake from total uptake.

Biotinylation and western blotting

Biotinylation was performed to determine the cell surface expression level of NET and impact of the co-expression of H_3R on NET surface trafficking. Cells were washed 48h after transfection, and incubated with sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate(sulfo-NHS-SS-biotin) (Pierce, Rockford, IL, USA). Biotinylated proteins were then isolated by neutravidin (Pierce, Rockford, IL, USA) binding. Total protein and membrane fraction were subjected to SDS-PAGE (5–20% gel, BioRad, Tokyo, Japan) and transferred to a PVDF membrane (GE Healthcare Biosciences, Buckinghamshire, UK). Membrane was immunoblotted with mouse anti-rNET monoclonal antibody (1:500, MAb Technologies, Stone Mountain, GA, USA) or rabbit anti-actin polyclonal antibody (1:200, Sigma-Aldrich Corporation, St.

Louis, MO, USA) followed by HRP-conjugated secondary antibodies, and detected by an autoradiographic film (GE healthcare Bioscience, Inc., Buckinghamshire, UK). The quantification of signals were conducted by using densitometry with NIH Image software as described previously [28].

Immunofluorescence

Regarding immunostaining, NET/CHO cells were grown on a BD Biocoat™ culture slide (Becton Dickinson Labware, Bedford, MA, USA) at 3.4×10^4 cells/well. Cells were washed with PBS containing Ca^{2+} and Mg^{2+}, and fixed with 4% paraformaldehyde for 10 min. Cells were then permeabilized by incubating with PBS containing 0.25% Triton X-100 for 5 min and blocked with 2% goat serum for 30 min. After that, cells were sequentially incubated with primary antibodies (rabbit anti-rH_3R polyclonal antibody, 1:1000, Millipore Corporation, Temecula, CA, USA; mouse anti-rNET monoclonal antibody, 1:1000) and secondary antibodies (Alxea 488 conjugated goat anti mouse antibody,1:1000, Alexa 594 conjugated goat anti rabbit,1:1000, Life Technology, Carlsbad, CA, USA). Immunofluorescent images were generated using a Keyence fluorescence microscope (KEYENCE BZ-9000, Keyence, Osaka, Japan).

Co-immunoprecipitation (Co-IP)

To investigate the presence of interaction between H_3R and NET, 200 μg of total cell extracts were subjected to immunoprecipitation with 50 μl of a 50% slurry of protein A sepharose 4 Fast Flow beads (GE Healthcare Biosciences, Buckinghamshire, UK) precoated with 1 μg antibody against rH_3R at 4℃ for 1h. Beads were washed with RIPA buffer (PBS containing 1% (v/v) Nonidet P40, 0.5% sodium deoxycholate and 0.1% SDS) and proteins that bound to the beads were then eluted with 2× sampling buffer (0.125 M Tris-HCl, 10% SDS, 20% glycerol, 0.02% bromophenol blue, 0.2 M DTT, pH 6.8). Following SDS-PAGE, immunoblotting was performed with an anti-rNET antibody (1:500) as described above.

Statistical analysis

Values are shown as mean ± SE. A Mann-Whitney's U test was used For comparisons between two groups. Analyses were performed using a one-way analysis of variance (ANOVA) with pair-wise comparisons followed by the Bonferroni method for comparisons of more than two groups. Significant level was set at 0.05.

Results

Effects of H_3R co-expression on NET uptake activity

Since NET functions as a reuptaker of released NE and terminates the neurotransmission, we initially determined whether the co-expression of H_3R had any effects on NET uptake activity. NET/CHO cells were transiently transfected with either H_3R, H_3R-antisense, or mock (pcDNA3) and uptake activity of NET was measured. We detected H_3R mRNA expression in H_3R transfected NET/CHO cells using RT-PCR (data not shown). NET uptake activity was significantly lower with the transfection of H_3R than with the mock (Fig. 1). On the contrast, when we transfected the vector with H_3R antisense sequence, no significant effect was observed in NET uptake activity (Fig. 1).

Effects of H_3R co-expression on NET membrane expression

NET is a transmembrane protein that functions only when it is expressed on cell membrane. Therefore, we investigated whether decreased uptake activity was accompanied by a reduction in membrane expression level of NET. As shown in figure 2A, membrane fraction contained only highly glycosylated form (mature form) of NET with a molecular weight of ~75kD, while total fraction contained both mature and immature forms (less glycosylated form, ~37kD and ~50kD). Expression of H_3R was detected both in the total and membrane fractions by western blotting with

specific antibody against H_3R (data not shown). In consistent with uptake assay results, surface biotinylated level was significantly lower in NET/CHO cells transfected with H_3R than in those transfected with mock (Fig. 2A, 2B). However, no significant difference was observed in total NET protein level between H_3R and mock (Fig. 2A, 2B).

We then examined the impact of H_3R overexpression on NET localization following H_3R transfection using immunocytochemistry. As shown in figure 3, NET was stained in green and H_3R in red. The expression of H_3R was observed only in the H_3R transfected NET/CHO cells (Fig. 3f). NET was evenly distributed throughout the cell transfected with mock (Fig. 3a-d). However, when H_3R was co-expressed, NET was accumulated into perinuclear granules, resulting in an uneven distribution of NET (Fig. 3e). Moreover, co-localization of NET and H_3R was observed when we overlaid NET and H_3R staining images (Fig. 3g).

Protein to protein interaction between H_3R and NET

The co-localization of NET and H_3R prompted us to determine whether NET and H_3R interacted with each other when co-expressed. NET/CHO cells transfected with H_3R or mock were lysed and immunoprecipitated with an antibody against either H_3R or NET. When immunoprecipitated with an antibody against H_3R, the presence of NET reactivity was detected (Fig. 4). On the other hand, NET reactivity was not

detected in the mock-transfected group following immunoprecipitation (Fig. 4).

Discussion

Monoamine transporters can be modulated through a number of mechanisms including substrate exposure, phosphorylation/dephosphorylation and protein to protein interaction [16, 29, 30]. Some binding partners for NET, including Hic3, PP2A, syntaxin 1A, 14-3-3 and neurokinin-1 receptor, have already been identified [16, 17]. These proteins are known to regulate NET function, trafficking, and membrane expression through protein-protein interactions. Here we have reported H_3R as a new binding partner for NET. The co-expression of H_3R in CHO cells stably expressing NET resulted in the co-localization of NET and H_3R, downregulated specific [^{3}H] NE uptake, and decreased the NET membrane expression level. Moreover, the results of co-IP experiments indicated the existence of a physical interaction between NET and H_3R.

Approximately 80% to 90% of the NE released from noradrenergic neurons is cleared through reuptake by NET [30]. The regulation of NET has important functional consequence, given the role of NE in control of emotion, cognition and blood pressure, among many other physiological functions. Previous studies implicated malfunctioned NET in the pathogenesis of OI and other diseases [1, 4, 5]. The results of the present study identified H_3R as an important component in regulating synaptic NE

levels, and thus, noradrenergic neurotransmission. Imamura and colleagues have demonstrated that activation of H_3R reduces NE release [20]. Together with our results that showed co-expression of H_3R suppresses NET membrane expression level and NET mediated uptake activity (Fig.1 and Fig. 2), we speculate that H_3R may modulate synaptic NE concentration by balancing the release and reuptake of NE. In consistent with our speculation, NE release is enhanced in H_3R KO mice while NE level is not changed [26, 31].

NET is located in the presynaptic terminal of noradrenergic neuron that controls the noradrenergic tone through reuptake of released NE [6]. In contrast to dopamine transporter (DAT) and serotonin transporter (SERT), which are localized to plasma membrane in both the axon terminal and somatodendritic compartment, electron microscopy studies demonstrated that NET is primarily found to be expressed in intracellular compartment of nerve terminal of the prefrontal cortex (PFC) [32]. The co-expression of H_3R with NET was shown to cause the intracellular accumulation of NET in NET/CHO cells (Fig. 3), thereby suggesting a possible mechanism for the regulation of NET trafficking in PFC, considering both NET and H_3R are shown to be widely expressed in the cortex [32, 33].

The regulation of NET by H_3R and their interaction extends the previous findings that demonstrated the regulation of monoamine transporters by G protein coupled receptors (GPCRs) through protein to protein interaction [17, 34, 35]. Previous reports have shown that SLC6 family members can be modulated by GPCRs. Dopamine D2 receptor (D_2R) interacts with DAT and positively modulates DAT

function both in cultured cells and rat synaptosomes. The regulation of DAT by D_2R is not affected in the presence of D_2R antagonist, suggesting that it is independent of D_2R activation [35]. SERT was also shown to be regulated by adenosine A3 receptor (A_3AR) in HEK293 cells [34]. In contrast, Zhu et al. showed that A_3AR agonist treatment increases surface expression of SERT and 5-HT uptake. More recently, Arapulisamy et al. reported that the neurokinin-1 receptor interacted and modulated NET function [17]. To the best of our knowledge, this is the first study to show the regulation of a monoamine transporter by a heteroreceptor. H_3R has been indicated to be a heteroreceptor in many non-histaminergic neurons, such as dopaminergic and serotoninergic neuron [19, 24]. Therefore, it is reasonable to suggest that the expression of H_3R also influences the function of respective transporters in order to control neurotransmission in these neurons. H_3R KO mice exhibit blunted responses to amphetamine, which mainly exerts its effects through DAT, indicating a possible role of H_3R in the modulation DAT [31]. Taken together, interactions between GPCRs and monoamine transporters may represent a general regulatory mechanism for monoamine transporter function and trafficking, although further efforts are needed to elucidate the physiological significance of these processes.

Conclusion

In summary, our results provide first evidence that NET can be modulated by the

heteroreceptor H_3R. The overexpression of H_3R together with NET downregulates NET membrane expression and uptake activity in NET/CHO cells. Furthermore, H_3R and NET co-localize in cytoplasm and show physical interaction when co-expressed. The regulation of NET by H_3R will provide opportunities for further research in understanding the influence of histamine in control of NE signaling.

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Figure legends

Figure 1 Effect of co-expression of H_3R on [^{3}H] NE uptake by NET in NET/CHO cells.

[^{3}H] NE accumulation was measured in NET/CHO cells transfected with equal amount of pcDNA3 (mock), H_3R, or H_3R-antisense DNA. Non-specific activity was determined by application of 100 μM cocaine. Data are given as mean±SE, and analyzed by one way ANOVA, followed with *post hoc* Bonferroni test. ***: $p<0.001$, between H_3R and mock or between H_3R and H_3R antisense ; n=8-9.

Figure 2 Effects of H_3R co-expression on NET membrane expression

NET/CHO cells transfected with mock or H_3R were biotinylated and biotinylated proteins were isolated by avidin binding. Total protein and membrane fraction were subjected to SDS-PAGE, followed by immunoblotting with NET antibody. Membrane fraction of NET are shown at ~75kD. β-actin immunoblots are shown for equal protein load (A). Quantification of western blots for total (mature plus immuature) and membrane (mature) portion of NET are shown in B. (n=4, Mann-Whitney's U test) **: $p<0.01$; n.s.: not significant.

Figure 3 Immunocytochemistry of NET and H_3R after mock or H_3R transfection in NET/CHO cells.

Cells were treated as described in "Materials and Methods". Panels a-b are pictures of

mock transfected NET/CHO cells which are devoid of H_3R expression. Panels e-f are pictures of H_3R transfected NET/CHO cells. Panels c and g are overlays of panel a, b and e, f, respectively. Panels d and h are differential interference contrast (DIC) pictures of the field shown above. Scale bar: 50μm.

Figure 4 Physical association between NET and H_3R

NET/CHO cells transfected with mock or H_3R were lysated with detergent and then incubated with protein A beads precoated with antibody against H_3R. Bound protein were eluted and subjected to SDS-PAGE, followed by immunoblotting with anti-NET antibody. IP: samples that underwent immunoprecipitation with indicated antibody; Input: samples that did not undergo IP.

Figure 1

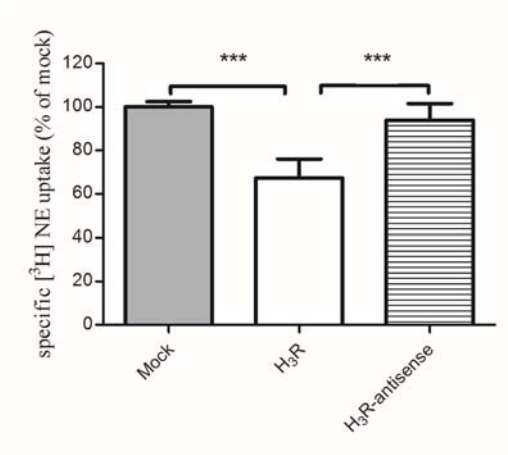
specific [3H] NE uptake (% of mock)
120
100
80
60
40
20
0
***
***
Mock
H3R
H3R-antisense

Figure 2

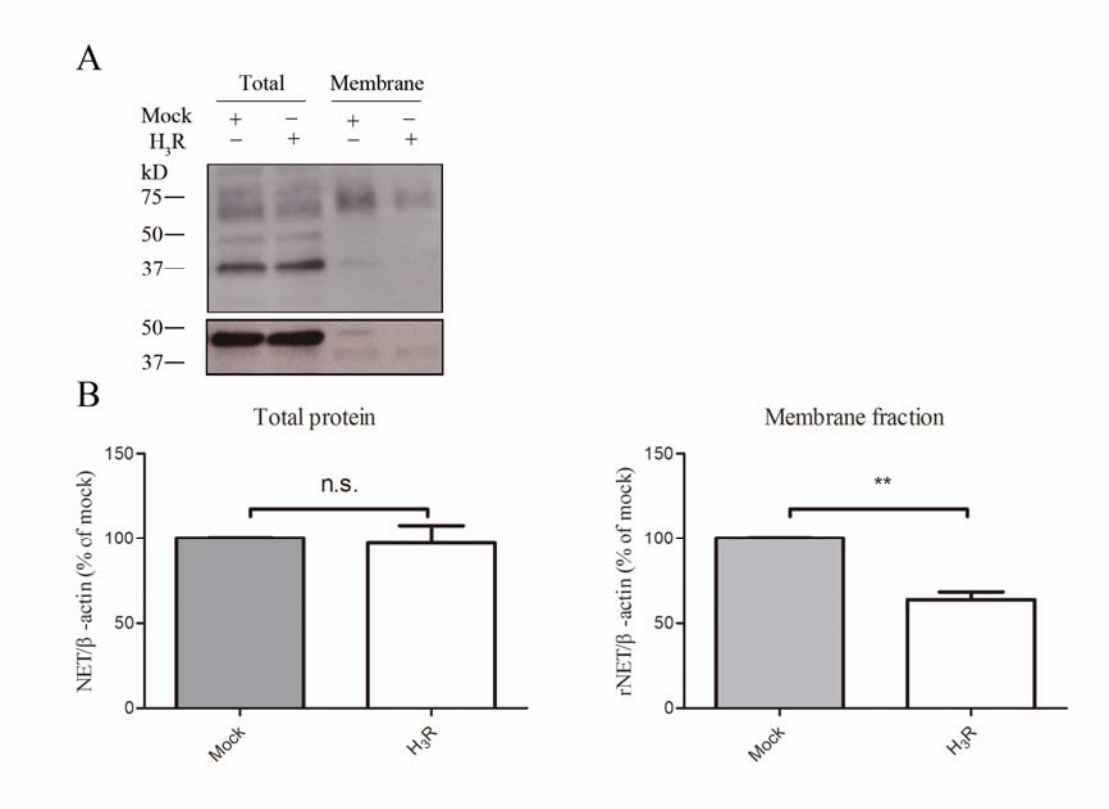
A
Total
Membrane
Mock
H_3R
kD
75
50
37
50
37
B
Total protein
n.s.
NET/β -actin (% of mock)
150
100
50
0
Mock
H_3R
Membrane fraction
**
rNET/β -actin (% of mock)
150
100
50
0
Mock
H_3R

Figure 3

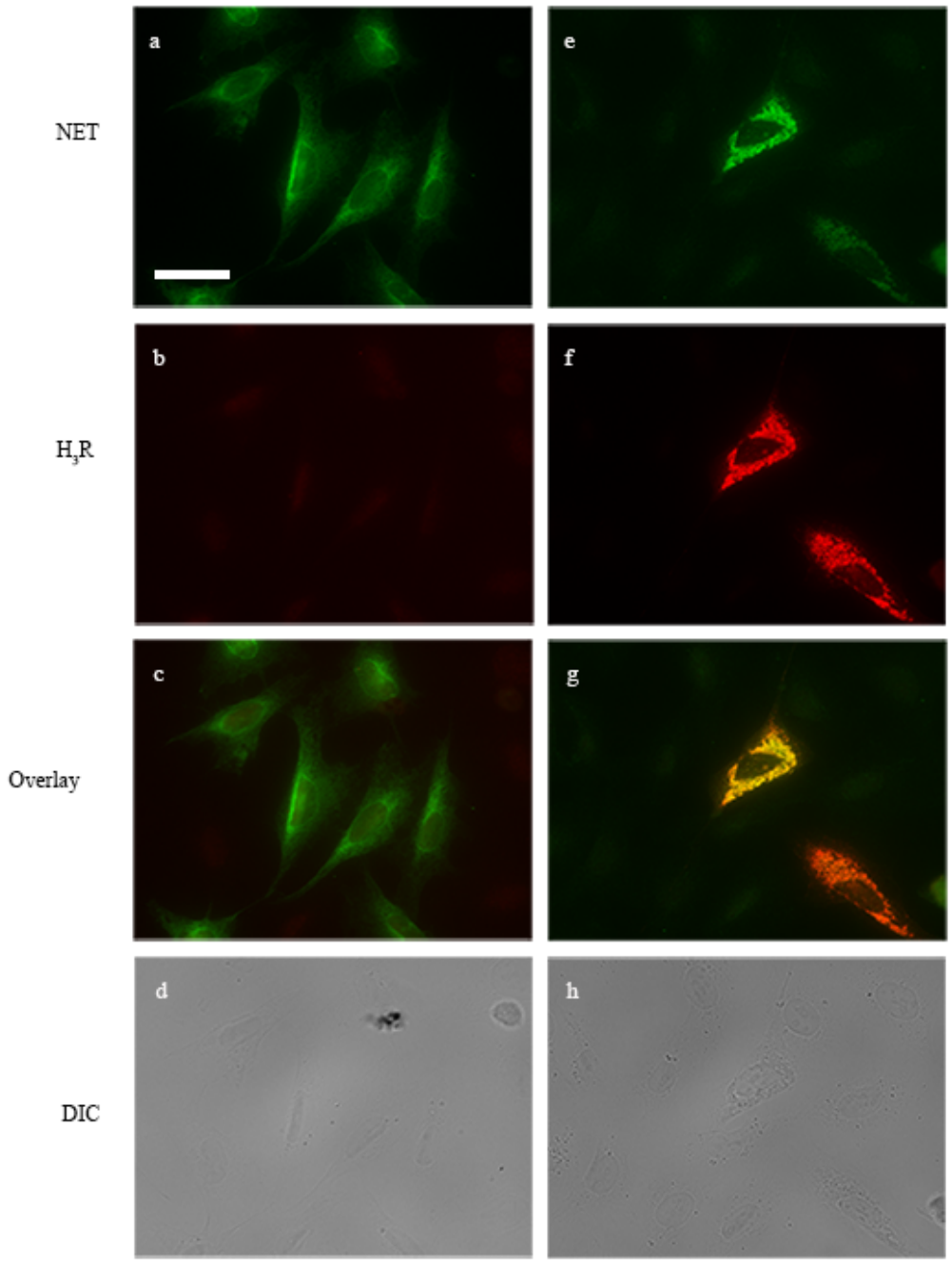

Figure 4