Biochemical and Biophysical Research Communications 415 (2011) 270–273

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Protein tyrosine phosphatase CD45 as a molecular biosensor of hydrogen peroxide generation in cell culture media

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ARTICLE INFO

Article history:
Received 28 September 2011
Available online 19 October 2011

Keywords:
Hydrogen peroxide
Oxidative stress
Cell culture media
Jurkat cells
Protein tyrosine phosphatase CD45
Reversible oxidation

ABSTRACT

We have designed a useful method of assessing reactive oxygen species generation in biological fluids. The novel assay utilizes tyrosine phosphatase CD45 as a biosensor of oxidative stress. Applying this new method, we examined oxygen species generation in the following cell culture media: RPMI 1640, DMEM, DMEM enriched with pyruvate and MEM. We discovered that the media (especially RPMI 1640) significantly reduced the activity of protein tyrosine phosphatase. The media-caused inactivation of CD45 was reversible after treatment with dithiothreitol being a powerful reducing agent. Interestingly, the media supplemented with catalase did not exhibit any inhibitory effect on CD45 activity which suggests a hydrogen peroxide-mediated mechanism of the enzyme inactivation. In addition to that, we assessed the impact of oxidative stress level on the activity of CD45 as measured in Jurkat cells cultured in RPMI 1640 either exposed or not exposed to the light of laminar flow cabinet fluorescent lamp. We found that Jurkat cells that were exposed to light displayed ca. 20% lower activity of CD45 than the cells protected against the light. The obtained results indicate that production of hydrogen peroxide in the medium leading to inhibition of CD45 was light-dependent, and that careful protection of cell culture media from the light may help to prevent the artifact in cell studies. Hydrogen peroxide, responsible for CD45 inactivation, can be generated in cell culture media after exposition to light due to photoreactive amino acids present in the media.

1. Introduction

Tissue culture techniques are applied in laboratories worldwide in order to study cellular metabolism, signal transduction, toxicity, and drug testing. Despite the universal utilization of cultured cells, scientists realize that they are just a vague approximation of physiological conditions. A chemical composition of cell culture media being different from the natural cellular environment, together with relatively high partial pressure of oxygen, and lack of efficient physiological oxygen defense mechanisms may lead to excessive generation of reactive oxygen species (ROS) in extracellular milieu. It has been demonstrated that ROS can be spontaneously produced in RPMI 1640, Dulbecco's modified Eagle medium and minimum essential medium [1]. It is worth mentioning, however, that cultured cells themselves are partly responsible for oxidative stress level in the medium [2,3]. There is growing evidence that certain compounds making up the cell culture medium undergo rapid chemical transformations to generate hydrogen peroxide [4,5]. These compounds are thiols, which are unstable in commonly used cell culture media (RPMI 1640, DMEM, MEM), and their autoxidation generates hydrogen peroxide. It concerns especially such compounds as reduced glutathione, which is a component of the RPMI 1640 medium or cysteine presents in DMEM, MEM, and RPMI 1640 [6]. Previous studies indicate that superoxide and hydrogen peroxide can be generated in cell culture media in a light-dependent manner [7]. Superoxide hardly penetrates into a cell, but hydrogen peroxide can easily cross cell membrane and cause damage to cellular biomolecules either directly or after conversion to even more deleterious hydroxyl radical [8]. Some cell culture media, e.g. DMEM, contain iron(III) nitrate, which after initial reduction may serve as a substrate for the Fenton reaction generating hydroxyl radical [9]. It is worth mentioning that hydrogen peroxide was found to inactivate protein tyrosine phosphatases (PTPs) [10,11] containing a vulnerable cysteine residue in the catalytic center that is essential for enzymatic activity [12,13]. Taking it all into consideration, we have proposed a novel method, utilizing CD45 (selected protein tyrosine phosphatase) as a sensitive hydrogen peroxide biosensor [14]. We examined and compared four popular cell culture media in terms of PTP activity. In addition to that, we

Abbreviations: PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; DMEM, Dulbecco's modified Eagle medium.

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doi:10.1016/j.bbrc.2011.10.033

measured the enzymatic activity of CD45 expressed in abundance in Jurkat cells [15]. In order to assess if reactive oxygen species production in cell culture media is light-dependent, we cultured cells exposed or not exposed to light (laminar cabinet fluorescent lamp, ~800 lux).

2. Materials and methods

2.1. PTP CD45 activity assay

Human recombinant CD45 protein tyrosine phosphatase was obtained from Calbiochem (San Diego, CA). Dulbecco's modified Eagle medium with L-glutamine (DMEM; Cat. No. D5796), DMEM containing 1 mM sodium pyruvate (Cat. No. D6546), MEM containing 1 mM pyruvate (Cat. No. M4526) and RPMI 1640 with L-alanyl-glutamine (Cat. No. R2405) were purchased from Sigma. DMEM containing sodium pyruvate and MEM were supplemented with L-glutamine. The stock solution of the enzyme was prepared in 50 mM Tris buffer, pH 7.4 and added into DMEM, DMEM containing sodium pyruvate, MEM, RPMI 1640 medium without any supplementation or supplemented with 900 μg/mL catalase or 1 and 10 mM sodium pyruvate (Sigma). The final concentration of CD45 in testing samples was 10 μg/mL. The solution of the enzyme in Tris buffer with 900 μg/mL catalase was as a control sample. Samples were incubated for 15 min at 37 °C and 1 mM synthetic substrate pNPP (Sigma) in 50 mM Tris buffer, pH 7.4 was added. After following 10 min of incubation, PTP activity was measured in 96-well microplates. PTP hydrolyzed pNPP to p-nitrophenol and inorganic phosphate. The production of yellow colored p-nitrophenol was assessed as an increase in absorbance at 405 nm using microplate reader Jupiter (Biogenet) and DigiRead Communication Software (Asys Hitech GmbH). Subsequently the 20 mM dithiothreitol (DTT) (Sigma) was added to each sample for 30 min and recovery of the enzyme activity was measured.

2.2. Cell culture conditions

The human Jurkat cell line, clone E6-1, was obtained from European Collection of Cell Culture (ECACC, UK). Cells were cultured at 37 °C in RPMI 1640 medium (powdered medium purchased from Sigma) dissolved in sterile water supplemented with 10% fetal bovine serum (Sigma), 100 μg/mL penicillin/streptomycin and 2 mM L-glutamine. Cultures were maintained at a density of 1×10^6 cells/mL.

2.3. Determination of PTP CD45 activity in Jurkat cells

Jurkat cells were incubated with or without (control) catalase or sodium pyruvate for 1 h at 37 °C. After incubation cells were rinsed with TBS, lysed (1×10^7 cells/mL) in 0.5% NP-40 pH 7.5, 25 μg/mL leupeptin, 25 μg/mL pepstatin, 2 μg/mL aprotinin, 1 mM PMSF (Sigma) and 100 μl of cell lysate was added to the wells of 96-well plate coated before with 8 μg/mL mouse anti-human CD45 antibody (R&D Systems) in PBS, incubated overnight at room temperature and washed with 0.05% Tween 20 in PBS. After adding lysate plates was placed on rocking platform at 30 rpm for 3 h at room temperature. Lysates were aspirated from the wells; the wells were then washed with 50 mM HEPES/0.5% NP-40, and 200 μM Tyrosine Phosphate Substrate DADEY(PO_3)LIPQQG (R&D Systems) in deionized water was added. Inorganic phosphate production was assessed using malachite green-molybdate binding reaction. Absorbance was measured using a microplate reader Jupiter with 620 nm filter.

2.4. Determination of PTP CD45 activity in Jurkat cells cultured in light or in darkness

Cells were cultured either in complete darkness (medium was prevented from light all the time) or partly exposed to light with total exposition time of ca. 3 h. The source of light was a laminar flow cabinet fluorescent lamp (>800 lux). After the third passage cells were suspended in fresh medium and incubated for 24 h at 37 °C. After incubation cells were rinsed with TBS, lysed (1×10^7 cells/mL) and CD45 activity was determined as described above.

2.5. Statistical analysis

All the experiments were performed at least three times. The results were expressed as mean ± S.E.M. Statistical analyses were performed using the combination of ANOVA and Tukey's test (GraphPad Prism Software v.4). Differences between the means were considered significant for $p < 0.05$.

3. Results

We examined four cell culture media: RPMI 1640, DMEM, DMEM containing 1 mM sodium pyruvate and MEM containing 1 mM pyruvate. We observed that enzymatic activity of protein tyrosine phosphatase CD45 in RPMI 1640 significantly decreased as compared to control in Tris buffer. The rate of inactivation (expressed as percent of control) was lower for DMEM, but the inhibitory effect was least for media already containing pyruvate (Fig. 1). After having measured the activity of CD45, the samples were treated with 20 mM DTT (reducing agent) in order to reverse medium-dependent inactivation. Our results indicate that media-caused loss of PTP activity may be almost completely restored after treatment with 20 mM DTT (Fig. 1).

We assessed the activity of CD45 in RPMI 1640 and DMEM with addition of sodium pyruvate. Treatment with 1 mM sodium pyruvate caused increase of CD45 activity, but only 10 mM pyruvate

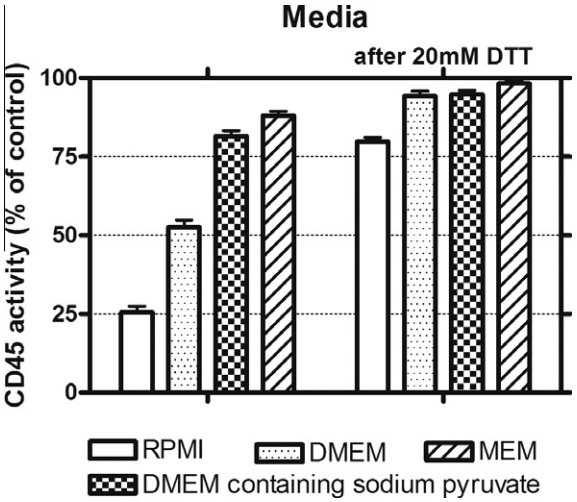

Fig. 1. The effect of cell culture media on enzymatic activity of PTP CD45 before and after treatment with 20 mM DTT. CD45 was incubated at 37 °C for 15 min in the following media: RPMI 1640, DMEM, DMEM containing 1 mM sodium pyruvate and MEM containing 1 mM pyruvate. The activity was measured 10 min after adding pNPP substrate using microplate reader at 405 nm. After assessment, 20 mM DTT was added and the recovery of enzymatic activity was measured after 30 min. The results were expressed as percent of activity of control sample (50 mM Tris buffer with 900 μg/mL catalase, pH 7.4). The data present mean ± S.E.M. from four separate experiments.

managed to entirely abolish the inhibitory effect of the media on enzymatic activity (Fig. 2).

We supplemented RPMI 1640 and DMEM with catalase, an enzyme decomposing hydrogen peroxide. In the presence of 900 μg/mL catalase in media as well as in a sample containing 20 μM hydrogen peroxide the inhibition of CD45 was almost completely attenuated (Fig. 2).

We observed no difference in enzymatic activity after incubation of CD45 in the media supplemented with or without 10% fetal bovine serum (data not shown).

In addition to that, we performed the PTP activity assay in Jurkat cells cultured in RPMI 1640 medium being the recommended one for this specific cell line. The activity of CD45 was

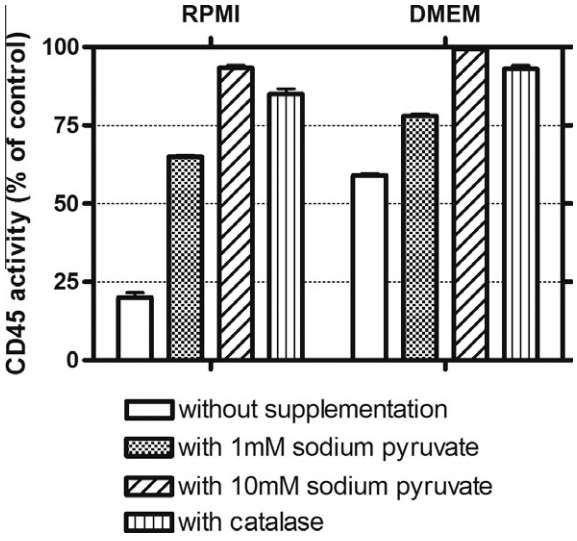

Fig. 2. Impact of media supplementation with sodium pyruvate and catalase on CD45 activity. Media were supplemented with 1 mM and 10 mM sodium pyruvate or with 900 μg/mL catalase and solution of CD45 was added for 15 min. pNPP hydrolysis to p-nitrophenol was measured 10 min after adding the substrate in microplate reader at 405 nm. The results were expressed as % of activity of control sample (50 mM Tris buffer with 900 μg/mL catalase, pH 7.4). Mean ± S.E.M., $n = 3$.

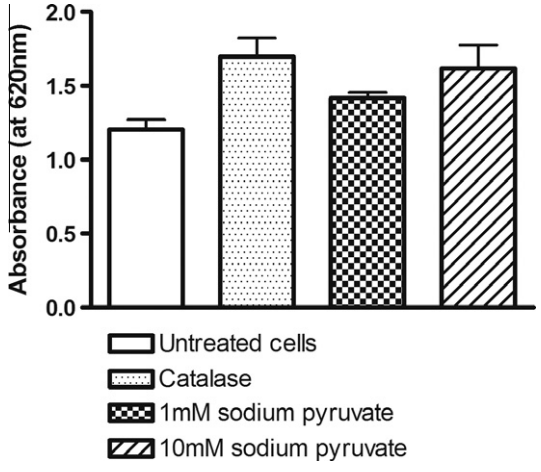

Fig. 3. The effect of additives to the culture media on activity of CD45 in Jurkat cells. Cells were incubated in fresh RPMI 1640 medium with or without 900 μg/mL catalase, 1 and 10 mM sodium pyruvate for 1 h at 37 °C. Cells were washed from medium and lysed (1×10^7 cells/mL); 100 μl of lysate was added to the wells of an anti-CD45 coated microplate. After 3 h incubation at room temperature the wells were washed three times with 50 mM HEPES/0.5% NP-40 and activity of CD45 was assessed using tyrosine phosphatase substrate DADEY(PO3)LIPQQG and malachite green-molybdate binding reaction for inorganic phosphate. The results were expressed as absorbance at 620 nm. Mean ± S.E.M., $n = 3$.

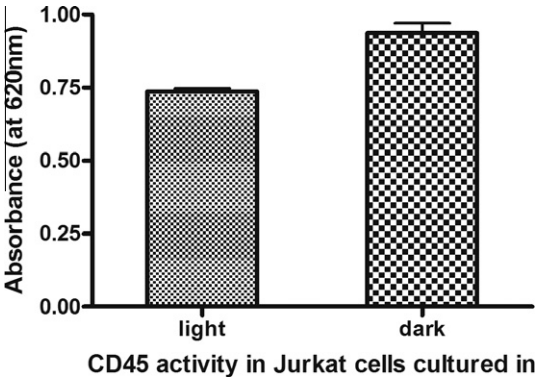

Fig. 4. The activity of CD45 in Jurkat cells cultured under exposure to light or in darkness. Cells were divided into two groups: one group comprised cells cultured under exposure to light, while the other one was comprised of cell culture and media protected from light. Total exposition time for cells culture exposed to light was ca. 3 h; the source of light was a laminar flow cabinet fluorescent lamp (>800 lux). After the third passage cells were incubated for 24 h in fresh medium at 37 °C. Then cells were lysed (1×10^7 cells/mL) and CD45 activity was determined using tyrosine phosphatase substrate DADEY(PO_3)LIPQQG followed by malachite green-molybdate binding reaction for detection of inorganic phosphate. The results were expressed as absorbance at 620 nm. The data from five independent experiments were present as mean ± S.E.M.

40% lower in untreated cells than in cells pretreated with 900 μg/mL catalase (Fig. 3). Incubation of cells with 1 mM sodium pyruvate caused 15% rise in PTP activity as compared to the untreated cells. Treatment with 10 mM sodium pyruvate resulted in more than 30% increase of CD45 activity (Fig. 3).

We assessed the activity of CD45 in Jurkat cells cultured with partial access to light or in complete darkness. We observed that cells cultured in the dark displayed 22% higher activity of CD45 as compared to the cells exposed to light (3 h under laminar flow cabinet fluorescent lamp, >800 lux) (Fig. 4).

4. Discussion

The main purpose of the present study was to design an assay, utilizing protein tyrosine phosphatase CD45 as biosensor of hydrogen peroxide in biological fluids, focusing on cell culture media. CD45 belongs to a large family of signal transduction enzymes, which dephosphorylate phospho-tyrosine residues in various biologically active proteins and is abundant in the leukocyte cell membrane playing important physiological role [16,17]. CD45 was chosen because it possesses redox-sensitive thiol in the catalytic center and as a thiol-containing protein, it is susceptible to oxidation, and reversible reduction that is possible under certain conditions [18]. Oxidation of thiolate suppresses the activity of PTPs. Thus, PTPs may act as extremely sensitive biosensors of oxidation–reduction potential [19]. We applied CD45 to examine four popular cell culture media. The obtained results demonstrate that cell culture media may surprisingly decrease the enzymatic activity of CD45, with RPMI 1640 displaying the strongest inactivation potential. It is crucial importance, as RPMI 1640 is the recommended medium for leukemic T cell lines, that are widely used in studies on CD45 function and regulation. We observed that the media containing pyruvate had much lower inhibitory impact on phosphatase activity as compared to the media devoid of pyruvate. Supplementation of cell culture media with 1 mM sodium pyruvate is a common practiced, not only to provide some additional energy source, but also to protect cells against hydrogen peroxide [20]. A previously documented [21] the level of hydrogen peroxide significantly decreased in the media formulated with pyruvate. We assessed the enzymatic activity of recombined CD45 in RPMI

1640 or DMEM supplemented with sodium pyruvate as well as CD45 in Jurkat cells pretreated with sodium pyruvate. Our results indicate that 1 mM concentration of sodium pyruvate, which was found to enhance cell viability [22], does not seem to sufficiently alleviate the inhibitory effect of the culture media on CD45 activity. Only 10 mM sodium pyruvate seemed to abolish the inhibition of the enzyme.

Treatment with DTT after inhibition of CD45 by the media resulted in almost 100% recovery of enzymic activity resembling the inhibitory effect of hydrogen peroxide that was completely reversed by thiol-active reductants. The catalytic cysteine, which exists as a thiolate anion, undergoes oxidation to sulfenic acid residue, which can be later reduced by DTT. However, excessive oxidation resulting in transformation to sulfinic or sulfonic acid residues is believed to be physiologically irreversible [23]. The ability of DTT to restore the activity of CD45 incubated in different media suggests that the catalytic cysteine undergoes oxidation to sulfenic acid, as observed in the case of the inhibitory effect of hydrogen peroxide. The fact that the media supplemented with catalase do not exhibit any inhibitory effect on PTP activity seems to prove a hydrogen peroxide-mediated mechanism of CD45 inhibition induced by different cell culture media.

We assessed the PTP activity in Jurkat cells cultured in RPMI 1640. We observed that cells pretreated for 1 h with catalase (900 μg/mL) showed 40% higher activity of CD45 than the untreated cells. Taking into consideration that hydrogen peroxide can easily cross cell membrane [8], we suppose that in absence of catalase, hydrogen peroxide is generated in the medium and may enter the cells inactivating protein tyrosine phosphatases.

There are many possible sources of hydrogen peroxide in cell culture media. Some components of the media, for example reduced glutathione and cysteine are able to generate hydrogen peroxide upon autoxidation processes [6]. Interestingly, some of cell culture media components may generate ROS in a light-dependent manner. It has been reported, that many amino acids present in cell culture media are photoreactive. Exposition to light may cause tryptophan, tyrosine, and histidine to generate superoxide anion undergoing further dismutation to produce hydrogen peroxide [24]. Previous studies demonstrated that both superoxide and hydrogen peroxide may be generated upon photoreactive transformation of riboflavin in RPMI 1640 as well as DMEM medium with folic acid enhancing the process [7]. The experiments performed with Jurkat cells either exposed to light or cultured in complete darkness proved that reactive oxygen species were generated in cell media causing further inactivation of CD45 in a light-dependent manner. Cells cultures exposed to light presented with 22% lower activity of CD45 as compared to cells protected from light.

Mammalian cell culture is a common laboratory technique being a useful alternative to animal model experiments. However, it should be taken into account that cell cultures may be exposed to higher concentrations of ROS, and specifically hydrogen peroxide, as compared to physiological *in vivo* conditions, and thus causing artifacts. It is of importance, especially while studying cell signaling pathways regulated by reversible oxidation–reduction processes. The obtained results suggest that protection of cell culture and the media from light, or effective supplementation with antioxidants, may help eliminate experimental artifacts caused by reactive oxygen species generated in cell culture media.

Acknowledgment

This work was supported by Grant No. N N401 029537 from the Polish National Science Center.

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