J. Biochem. 2011;149(2):171–176 doi:10.1093/jb/mvq129

JB THE JOURNAL OF BIOCHEMISTRY

Inhibitory effect of extracellular histidine on cobalt-induced HIF-1α expression

Received August 16, 2010; accepted October 19, 2010; published online October 29, 2010

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Cobalt chloride ($CoCl_2$) can mimic hypoxia in inducing hypoxia-inducible factor 1 (HIF-1). Several cultured cells were examined for susceptibility to $CoCl_2$ in DMEM, MEM and RPMI 1640 medium. Here we report that HIF-1α expression of mammalian cells by $CoCl_2$ was largely dependent on the culture medium. HIF-1α protein and hypoxia response element (HRE)-dependent reporter activity were strongly induced in RPMI 1640 but not in DMEM in several cultured cells including MCF-7, a human breast cancer cell line. Analysis of causal nutrients has revealed that histidine, which is contained richer in DMEM, acts as the inhibitory nutrient for cobalt-induced HIF-1α expression of MCF-7 cells in DMEM. D-Histidine also inhibited the HIF-1α activity at the same level as L-histidine, suggesting that sequestration of free cobaltous ion by chelation with histidine was the cause of the inhibition. These results demonstrate that selection of the culture medium must be considered with caution in cell culture experiments using $CoCl_2$ as a hypoxia-mimetic reagent.

Keywords: chelate formation/chemical hypoxia/cobalt chloride/histidine/hypoxia-inducible factor.

Abbreviations: $CoCl_2$, cobalt chloride; HIF, hypoxia-inducible factor; HLF, HIF-1α-like factor; HRE, hypoxia response element; H_2DCFDA, 2,7-dichlorodihydrofluorescein diacetate; MnTMPyP, manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride; NAC, *N*-acetyl-L-cysteine; PHD, HIF prolyl hydroxylase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

Cobalt chloride ($CoCl_2$) has been widely used as a chemical mimetic of hypoxia. Addition of $CoCl_2$ to the culture medium of mammalian cells activates hypoxia-inducible factor 1 (HIF-1), a crucial transcription factor for hypoxic gene regulation (*1–3*). HIF-1 is composed of an oxygen-sensitive HIF-1α subunit and a constitutive Arnt (HIF-1β) subunit. The HIF-1α subunit is degraded in normoxia by the ubiquitin-proteasome system after hydroxylation by HIF-prolyl hydroxylases (PHDs) and subsequent ubiquitination (*1–3*). In hypoxia, HIF-1α is stabilized, translocated into the nucleus and dimerized with Arnt to activate various hypoxia-responsive genes such as the vascular endothelial growth factor (VEGF) and erythropoietin genes. For the induction of hypoxic response in cultured cells, $CoCl_2$ was used at different concentrations ranging from ~0.05 to 1 mM. The underlying mechanism of cobalt action to induce chemical hypoxia is not fully elucidated. There are several hypotheses to explain its action. Inhibition of PHD activity by replacing the non-haem iron in the active site of PHDs stabilizes HIF-1α (*4*, *5*). Inactivation of PHDs by depleting intracellular ascorbate, which could keep oxidation state of the iron of PHDs, stabilizes HIF-1α (*6*). Direct binding of cobaltous ion to HIF-1α prevents its modification by PHDs (*7*). Recently several reports, including our own, have suggested that cobaltous ion acts as a generator of reactive oxygen species (ROS), leading to HIF-1α activation via PI3-kinase/Akt (*8–10*).

Present study shows that cobalt-induced HIF-1α expression of cells is highly dependent on the culture medium and that the histidine level in the medium is a key determinant for the response.

Materials and Methods

Cell culture and DNA transfection

Human breast cancer cell line MCF-7 and human neuroblastoma cell line IMR-32 were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. Mouse neuroblastoma—-rat glioma hybrid cell line NG-108-15 was kindly provided by Dr Fukunaga. MCF-7 and IMR-32 cells were cultured in DMEM, MEM or RPMI 1640 (RPMI) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum. NG-108-15 cells were cultured in DMEM or RPMI with 10% fetal bovine serum and sodium hypoxanthine (0.1 mM) + aminopterin (0.4 μM) + thymidine (16 μM) (HAT) supplement. DNA transfection was performed by the calcium–phosphate precipitation (*11*) and by lipofection using the transfection reagent (TransFast, Promega, Madison, WI, USA). After 4 h of transfection, medium was changed and $CoCl_2$ was added. The assay of luciferase activity was described previously (*11*). *N*-acetyl-L-cysteine (NAC) and manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) were purchased from Sigma-Aldrich (St Louis, MO, USA) and Calbiochem (San Diego, CA, USA), respectively.

Western blotting

Nuclear extracts were prepared as described previously (*10*). A total of 10 μg of protein was loaded on an 8% SDS–polyacrylamide gel. HIF-1α and HIF-1α-like factor (HLF, also known as HIF-2α) were detected using the ECL Western Kit (GE Healthcare, Little Chalfont, UK) with an anti-HIF-1α monoclonal antibody and an anti-HLF monoclonal antibody (Novus Biologicals, Littleton, CO, USA).

RT–PCR

Total RNA was prepared from cultured cells and cDNA was synthesized. PCR was performed as described previously (*10*). Bands were

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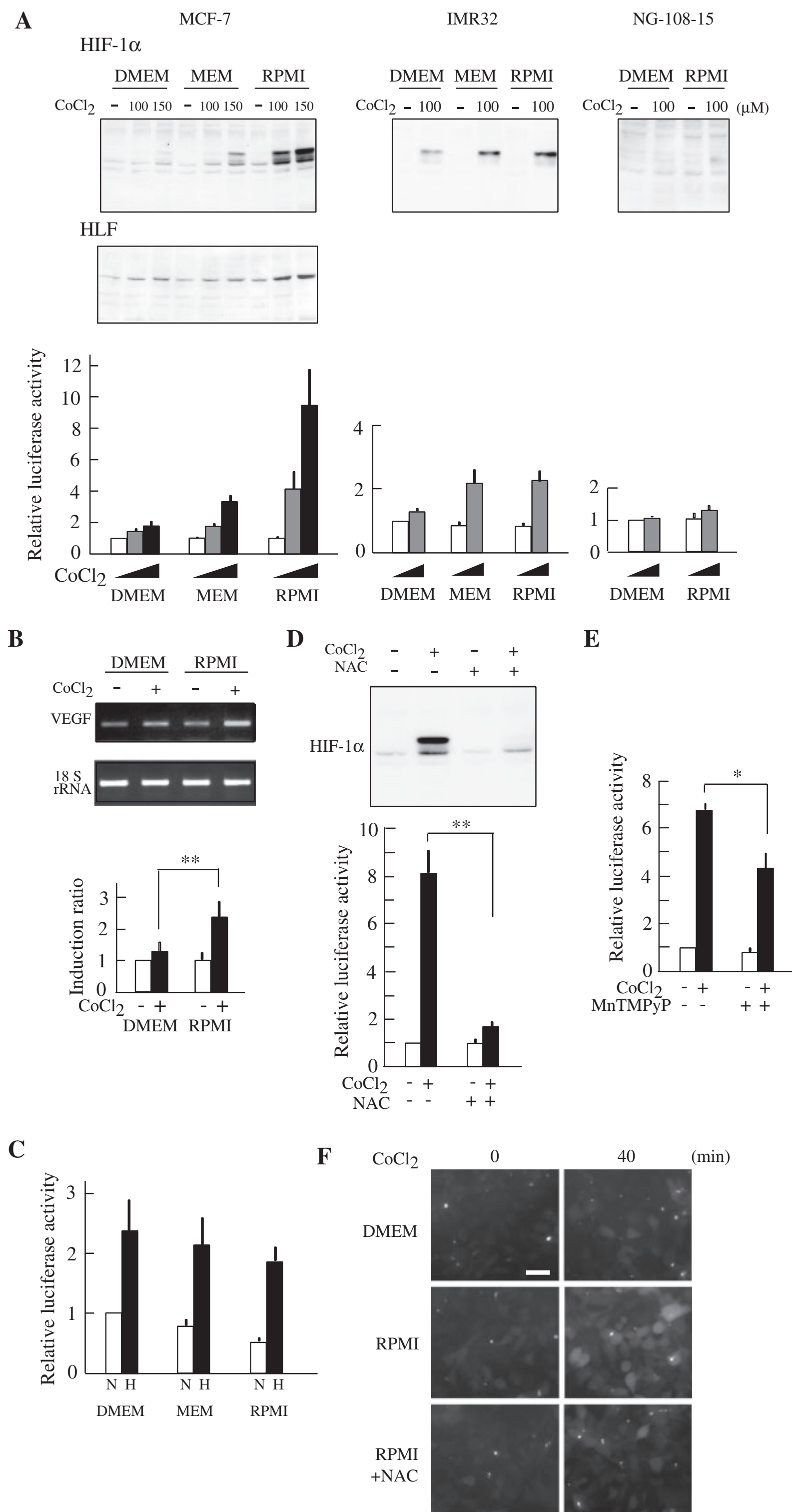

Fig. 1 Dependence of $CoCl_2$-induced HIF-1α expression on culture medium. (A) Induction of HIF-1α and HRE-dependent reporter activity by $CoCl_2$ in MCF-7, IMR-32 and NG-108-15 cells cultured in DMEM, MEM and RPMI. DMEM, MEM and RPMI contain 271, 271 and 97 μM histidine, respectively. MCF-7, IMR-32 and NG-108-15 cells were treated with $CoCl_2$ for 6 h. After the treatment, nuclear extracts were prepared, and HIF-1α protein was detected by western blotting using a monoclonal anti-HIF-1α antibody. HLF protein in the nuclear extracts of MCF-7 cells was also determined using a polyclonal anti-HLF antibody. After 4 h of transfection of the reporter plasmid, cells were treated with $CoCl_2$ for further 20 h, and cell lysates were prepared for reporter assay. The open, grey and filled bars indicate 0, 100 and 150 μM $CoCl_2$, respectively. (B) Induction of VEGF mRNA in MCF-7 cells cultured in DMEM and RPMI. After 6 h of $CoCl_2$ (150 μM) treatment, total RNA was extracted and VEGF mRNA was quantified by RT–PCR. A representative result of agarose gel electrophoresis is shown. 18 S ribosomal RNA was used as a control. (C) Induction of HIF-1α by hypoxia. After transfection of reporter plasmids, cells were incubated under hypoxic conditions (1% O_2) for 20 h, and luciferase activity was determined as in (A). (D and E) Inhibition of $CoCl_2$-induced HIF-1α expression by NAC (D) and MnTMPyP (E). MCF-7 cells were treated with 1 mM NAC or 100 μM MnTMPyP 1 h before $CoCl_2$ treatment. Western blotting and assay of luciferase activity were performed as described in (A). (F) $CoCl_2$-induced ROS production in MCF-7 cells cultured in DMEM or RPMI. MCF-7 cells were incubated with H_2DCFDA for 30 min in RPMI and then incubated in the indicated medium with 150 μM $CoCl_2$ in the presence or absence of 1 mM NAC. The intracellular levels of ROS were detected by measuring the DCF fluorescence. *$P<0.05$, **$P<0.01$.

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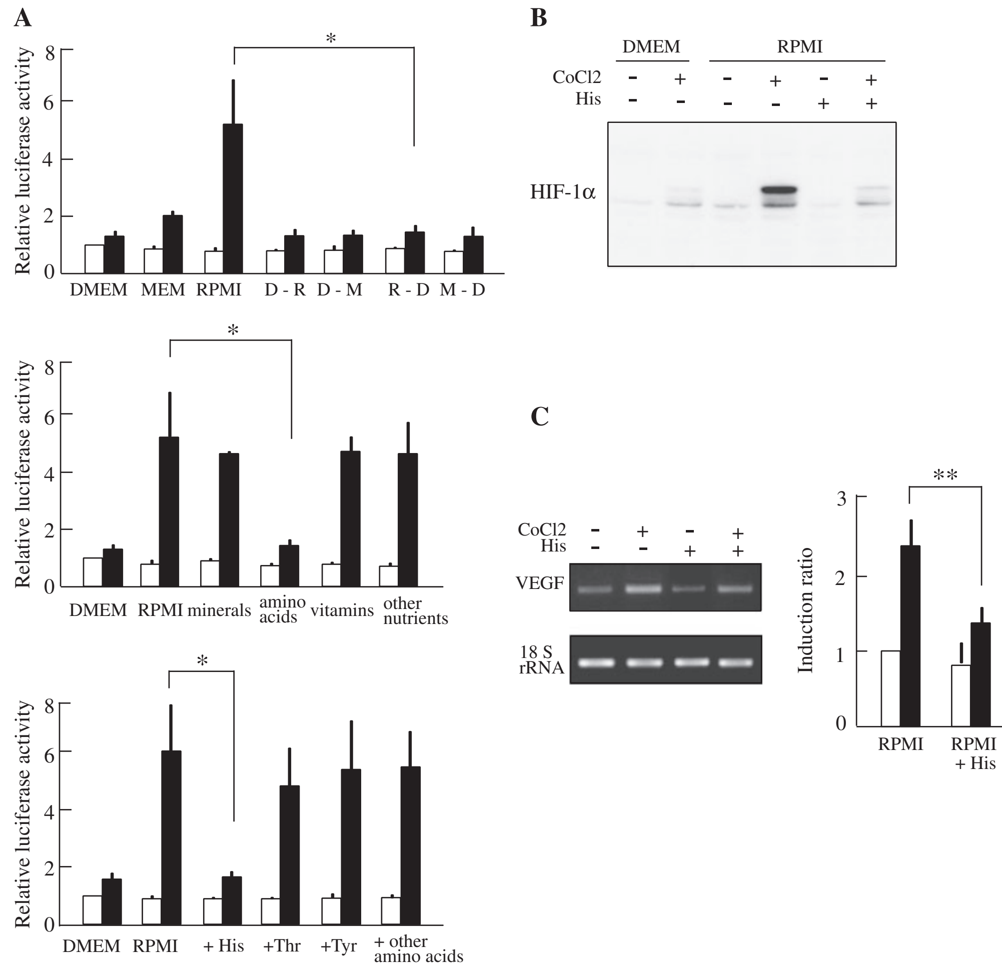

Fig. 2 Identification of nutrients responsible for repression of $CoCl_2$-induced HIF-1α expression in DMEM. (A) Identification of inhibitory nutrients in DMEM. MCF-7 cells were transfected with HRE-dependent reporter plasmid. After 20 h of incubation, cell lysates were prepared and luciferase activity was assayed as shown in Fig. 1. Filled and open bars represent luciferase activity in the presence or absence of $CoCl_2$, respectively. D–R and D–M indicate DMEM supplemented with excess amounts of nutrients contained in RPMI and MEM, respectively. R–D indicates RPMI supplemented with excess amounts of nutrients contained in DMEM. M–D indicates MEM supplemented with excess amounts of nutrients contained in DMEM. (Upper panel) Excess amounts of nutrients were grouped into four categories, minerals, amino acids, vitamins and other nutrients, and separately added to RPMI for reporter assay. (Middle panel) Amino acids were divided into histidine (final concentration, 271 μM), threonine, tyrosine and other amino acids and separately added to RPMI for reporter assay. (Lower panel) (B) Repression of $CoCl_2$-induced HIF-1α expression by histidine. Concentrations of histidine are 271, 97 and 271 μM in DMEM, RPMI and RPMI + His, respectively. After 4 h of cobalt treatment (150 μM), nuclear extracts were prepared and used for western blotting. Proteins were resolved in a 8% polyacrylamide gel, transferred onto nitrocellulose membrane. HIF-1α protein was visualized by using anti-HIF-1α monoclonal antibody. (C) Inhibition of VEGF mRNA expression by histidine. Histidine was added into RPMI at a concentration equal to DMEM (271 μM). Six hours after cobalt treatment (150 μM $CoCl_2$), total RNA was extracted and VEGF mRNA was quantified by RT–PCR. A representative result of agarose gel electrophoresis is shown. 18 S ribosomal RNA was used as a control. $^{*}P < 0.05$, $^{**}P < 0.01$.

quantified by using Image J software. Primer sequences used for 18 S rRNA are 5′-GGT CTG TGA TGC CCT TAG ATG TCC-3′ (forward) and 5′-GTT CGA CCG TCT TCT CAG CGC T-3′ (reverse) and sequences for VEGF are 5′-GAA CTT TCT GCT GTC TTG GGT GCA T-3′ (forward) and 5′-GGT CTG CAT TCA CAT TTG TTG TGC TG-3′(reverse).

Detection of ROS production

MCF-7 cells were plated onto 35 mm glass-bottom dishes at 1×10^6 cells per dish. After incubation for 24 h, cells (~90% confluent) were treated with 10 μM 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Wako Pure Chemical Industries, Osaka, Japan) for 30 min in RPMI, washed and incubated with RPMI or DMEM, and then treated with 150 μM $CoCl_2$ in the presence or absence of 1 mM NAC. Fluorescence images were obtained with an Olympus IX71 fluorescence microscope.

Statistical analysis

Data are given as mean ± standard deviation, with the number of the experiments indicated. Statistical significance was established by Student *t*-tests.

Results and Discussion

As shown in Fig. 1A, HIF-1α and HLF protein was strongly induced in MCF-7 cells in response to $CoCl_2$ when cultured in RPMI but not in DMEM. Difference of cell growth or cell viability in the cell culture conditions between RPMI and DMEM medium in the presence of $CoCl_2$ was not observed (Supplementary Fig. S1). Inducing effect of $CoCl_2$ was weak in MEM. According to the increment of HIF-1α expression, hypoxia response element (HRE)-dependent reporter activity was increased. Similar culture medium-dependent accumulation of HIF-1α protein by $CoCl_2$ was observed in IMR-32 cells, although induction of HIF-1α protein was weaker than that in MCF-7 cells. In NG-108-15 cells the induction of HIF-1α and reporter activity by $CoCl_2$ was negligible even when cultured

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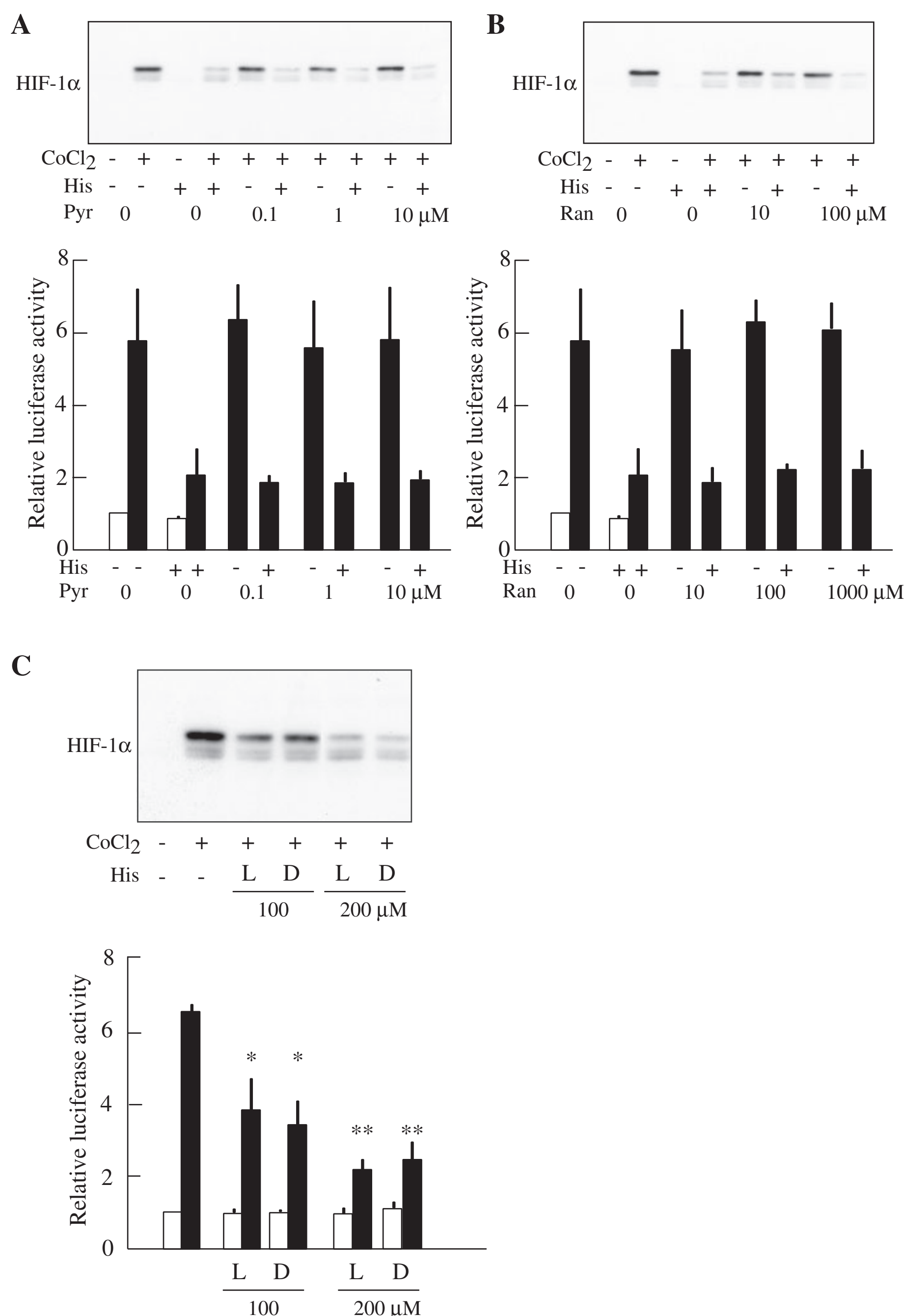

Fig. 3 Inhibition of cobalt-induced HIF-1α expression by D-histidine. (A and B) MCF-7 cells were treated with pyrilamine (A) and ranitidine (B) for 6 h in the presence or absence of 150 μM $CoCl_2$, and HIF-1α protein were determined by western blotting as shown in Fig. 1. Luciferase reporter activity was also determined 20 h after transfection. Cells were treated with pyrilamine or ranitidine 1 h before the addition of $CoCl_2$. (C) Repression of cobalt-induced HIF-1α expression by D-histidine. L- and D-histidine were added to RPMI to give a concentration of 100 and 200 μM. After 4 h of cobalt treatment (150 μM), nuclear extracts were prepared and used for western blotting. Whole cell extracts of MCF-7 cells were prepared 6 h after $CoCl_2$ treatment, and used for western blotting. Proteins were resolved in an 8% polyacrylamide gel, transferred onto nitrocellulose membrane. HIF-1α protein was visualized by using anti-HIF-1α monoclonal antibody. Filled bars and open bars represent luciferase activity in the presence or absence of $CoCl_2$, respectively. $^*P<0.05$, $^{**}P<0.01$.

in RPMI. In accordance with the HIF-1α expression, VEGF mRNA was induced by $CoCl_2$ in RPMI-cultured MCF-7 cells more strongly than that in DMEM (Fig. 1B). In contrast to $CoCl_2$ induction, HRE-dependent reporter activity was induced to a similar extent by hypoxia in MCF-7 cells cultured in DMEM, MEM and RPMI (Fig. 1C). In order to examine involvement of ROS in the induction of HIF-1α by $CoCl_2$, MCF-7 cells were treated with NAC (Fig. 1D). Induction of HIF-1α was completely repressed by NAC, suggesting that cobaltous ion functioned as a ROS generator. Since inhibition by MnTMPyP, a superoxide dismutase mimetic, was weak in the reporter activity, hydrogen peroxide but not superoxide anion may be involved in the activation of HIF-1α (Fig. 1E). Addition of catalase to the culture

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medium showed no effect on the induction of HIF-1α, suggesting that ROS generation by $CoCl_2$ occurred within the cells (data not shown). In addition, we examined cobalt-induced ROS generation by staining cells with the oxidant-sensitive dye, H_2DCFDA, in MCF-7 cells. In accordance with the HIF-1α expression, ROS production was strongly induced when cultured in RPMI but not in DMEM (Fig. 1F). Treatment with NAC largely attenuated the ROS production.

We examined the nutrients in DMEM that repress HIF-1α induction in MCF-7 cells. DMEM is a richer medium than RPMI in most of the nutrients. Thus, excess amounts of richer nutrients contained in DMEM were added to RPMI for reporter assays. As shown in Fig. 2A, addition of the nutrients repressed the induction of reporter activity by $CoCl_2$. In a reverse experiment using DMEM to which nutrients present richer in RPMI were added, no induction of reporter activity was found, suggesting that no causal nutrients in the added nutrients (Fig. 2A). The nutrients added to RPMI were divided into four groups, minerals, amino acids, vitamins and other nutrients, and separately added to RPMI. The resultant culture media were used for reporter assays. Causal nutrients were found in the group of amino acids (Fig. 2A). Further investigation of causal amino acids finally identified histidine as the causal nutrient. In the histidine-added RPMI, the reporter activity was decreased to the level of negative control of cells cultured in DMEM (Fig. 2A). In accordance with the result, HIF-1α protein levels were decreased to those in DMEM (Fig. 2B). Induction of VEGF mRNA was also significantly decreased by the addition of histidine to RPMI (Fig. 2C).

We previously reported that increment of cAMP levels repressed the expression of HIF-1α in PC12 cells (*12*). Histamine, a metabolite of histidine, could increase intracellular cAMP levels after binding to histamine H1 and H2 receptors, which are G-protein coupled receptor proteins (*13*, *14*). We examined this possibility that increased levels of extracellular histidine could enhance cAMP levels to decrease HIF-1α expression in MCF-7 cells. Two-types of histamine blockers, pyrilamine and ranitidine, H1 and H2 blockers, respectively, showed no effect on the HIF-1α level and reporter activity, thus ruling out action via an indirect autocrine pathway (Fig. 3A and B). It is known that cobaltous ion strongly binds to histidine to form a chelate complex with a mole ratio of one mole of cobaltous ion to two moles of histidine (*15*, *16*). In order to examine whether this chelate complex formation is related to HIF-1 inhibition by histidine, we added D-histidine instead of L-histidine to RPMI and examined HIF-1α expression in MCF-7 cells. D-Histidine has the same potential to form chelate complex with cobaltous ion as L-histidine, but is not expected to have biological activity that L-histidine has. Addition of D-histidine attenuated cobalt-induced HIF-1α expression with a dose-dependency similar to L-histidine (Fig. 3C). HRE-dependent luciferase activity was decreased in parallel with the decrease in HIF-1α protein levels. These results strongly suggest that loss of HIF-1α induction by $CoCl_2$ found in DMEM-cultured MCF-7 cells was caused by a decrease in free cobaltous ion due to chelation with histidine in the medium.

In addition to the activity to induce chemical hypoxia, $CoCl_2$ has harmful effects of heavy metals that inactivates protein functions and reduces cell viability. Those effects of $CoCl_2$ are obviously concentration-dependent, and it is therefore preferable to reduce $CoCl_2$ concentration as low as possible in cobalt-stimulated hypoxia experiments of cells. This study strongly suggests that selection of culture medium, especially low-histidine medium, is important to reduce effective concentrations of $CoCl_2$ used for hypoxia experiments, because histidine concentrations in the culture medium are considerably different. These observations implicate that slight differences in nutritional conditions intricately affect cell response to $CoCl_2$-induced hypoxia in a cell type-specific manner.

Supplementary Data

Supplementary Data are available at *JB* Online.

Acknowledgements

We are grateful to Dr Fukunaga (Tohoku University) for the generous gift of NG108-15 cells.

Funding

Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interest

None declared.

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