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Effect of Saliva, Epigallocatechin Gallate and Hypoxia on Cu-induced Oxidation and Cytotoxicity

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Abstract. *We have previously reported that contact with copper (Cu) induced immediate cell death via an oxidation-involved mechanism in human promyelocytic leukemic HL-60 cells, whereas contact with other metals (Au, Ag, Pd) produced no discernible effect. In the present study, we investigated the conditions under which Cu-induced oxidative stress can be reduced. Contact with a Cu plate in the absence of cells enhanced the rate of consumption of cystine to the greatest extent, followed by that of methionine and histidine. Under hypoxic conditions, the consumption of all these amino acids was significantly reduced. On the other hand, the addition of saliva slightly, but not significantly, reduced the amino acid oxidation. The addition of epigallocatechin gallate (EGCG) slightly, but significantly reduced the consumption of cystine and histidine. The inhibitory effect of EGCG on the methionine consumption was more prominent, especially at higher concentrations. The Cu-induced cell death was significantly inhibited when freshly-prepared human gingival fibroblasts were incubated under hypoxic conditions. The present study demonstrates for the first time that the Cu-induced oxidation and cell death were effectively alleviated under hypoxic conditions.*

Dental alloys have been reported to induce allergic reactions in the oral cavity, though infrequently (1), which may be related to the stimulated release of metal ions from the alloys under acidic oral environments caused by inflammation, bacterial infection, and intake of soft drinks and coffee (2, 3).

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Metal ions may be incorporated into cells, possibly *via* metal transporter-mediated endocytosis (4, 5). Although numerous studies have shown cytotoxic activity and tissue-damaging activity of metal extracts (6-8), no detailed study of cytotoxicity induced by the direct contact with metals has been reported. We have recently constructed an assay system to investigate the interaction between metals and cultured cells (9). Using this system, we found that the direct contact of cells with a copper (Cu) plate induced rapid, non-apoptotic cell death, characterized by a smear pattern of DNA fragmentation, minor caspase activation, loss of the membrane barrier, cytotoplasmic damage prior to nuclear damage, maintenance of cell surface microvilli, and vacuolization in the human promyelocytic leukemic cell line (HL-60), whereas other metals, such as gold, silver and palladium, were essentially inactive (9). It is very important to explore the method by which the cytotoxicity of Cu can be reduced. Here, we investigated the effect of three oral environmental factors: saliva, hypoxic conditions and epigallocatechin gallate (EGCG, a popular green tea polyphenol) on Cu-induced oxidative stress. The changes in the consumption rate of easily oxidizable amino acids in the culture medium was monitored without cells, and the viability of cultured human gingival fibroblast (HGF) at different oxygen concentrations after contact with Cu plate was determined.

Materials and Methods

Materials. The following metals, chemicals and reagents were obtained from the indicated companies: Cu plate (99.99%, 20x20x0.5 mm) (Tokuriki Honten, Co, Japan); fetal bovine serum (FBS), trichloroacetic acid (TCA) (Wako Pure Chem Co, Tokyo, Japan).

Polishing of metal plate surface. The Cu plates were polished using alumina slurry water (micropolish, Buehler) down to 0.05 μm particle size. After polishing, their surfaces were examined using scanning electron microscope (JSM-6360LV, JEOL, Japan) to confirm the consistency of surface smoothness.

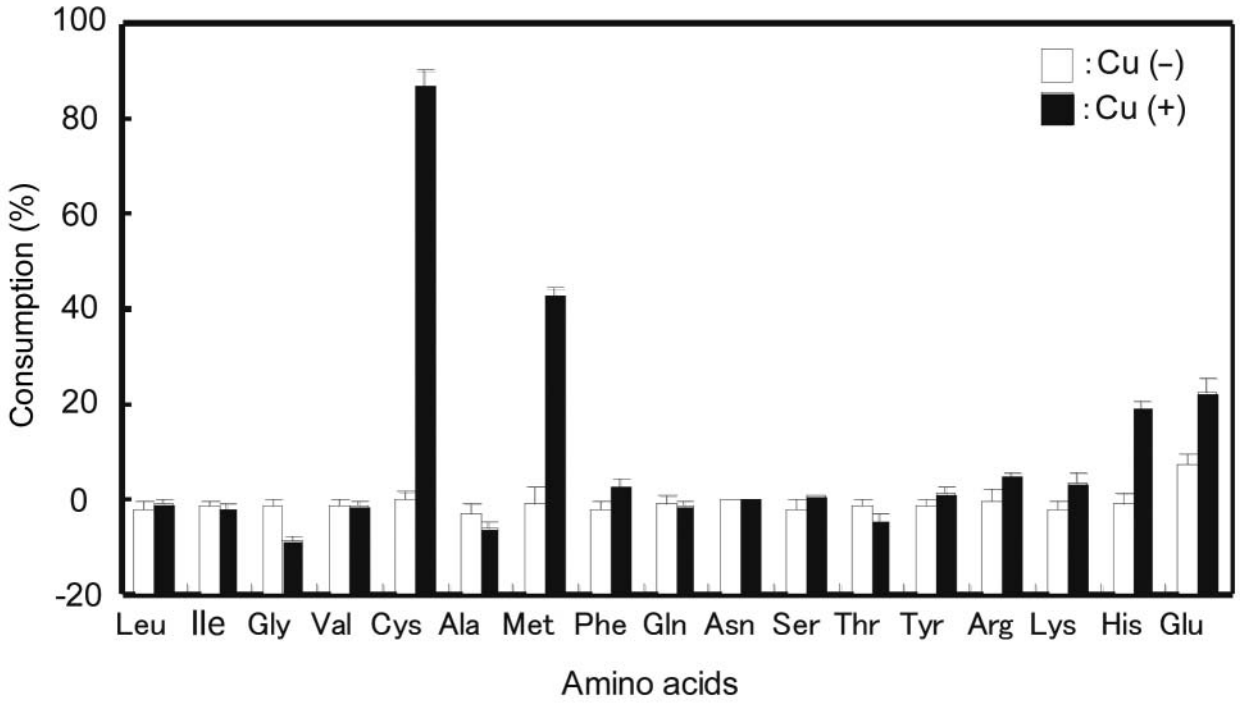

Figure 1. *Effect of contact with a Cu plate on amino acid consumption. Five hundred μL of culture medium (DMEM+10%FBS) was inoculated onto a plastic plate or onto a Cu plate, and then incubated for 0 or 30 min under normoxic condition (5%CO_2, 95% air). The consumption rate (%) was calculated as described in Materials and Methods. Each value represents the mean±S.D. from three independent experiments.*

Cell culture. HGF cells were prepared from periodontal tissues, according to the guideline of the Meikai University Ethic Committeee (No. 0206), after obtaining informed consent from the patients, and were cultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified normoxic 5% CO_2 atmosphere (Ikemoto Rika Kogyo, Tokyo). HGF cells were harvested by detaching them from the culture plate with 0.25% trypsin-0.025% EDTA-Na in phosphate-buffered saline without Mg and Ca [PBS(–)] and subcultured at a 1:4 split ratio once a week, with one medium change in between. Since HGF cells have a limited lifespan, ceasing proliferation at a 20 population doubling level (PDL) (10), the cells at 9-12 PDL were used in the present study.

Cytotoxicity of direct contact with Cu plate. HGF cells were trypsinized and resuspended at a cell desity of $2x10^6$ /mL in fresh medium. Five hundred μL of HGF cells ($2x10^6$ /mL) in DMEM + 10% FBS were inoculated on the Cu plate (in a 3.5 cm dish), and incubated for 30 min at 37˚C under normoxic (5% CO_2, 20% O_2) or hypoxic conditions (5% CO_2, 1% O_2) (Hypoxic incubator APM-30D, Astec, Tokyo). Cells were recovered from the metal plates by gentle pipetting. The viability of the cells was determined by cell counting with a hemocytometer after 0.15% trypan blue dye staining.

Determination of the changes in extracellular free amino acid. DMEM+10%FBS was mixed without or with saliva (50%) or EGCG (0.1 or 3.0 mM). Five hundred μL of these media were incubated on Cu plates for 30 min under normoxic (5% CO_2, 20% O_2) or hypoxic conditions (5% CO_2, 1% O_2). Culture supernatant (medium fraction) obtained after centrifugation (1,500 xg, 3 min) was mixed with an equal volume of 10% TCA, and stood on ice for 30 min. After centrifugation for 5 min at 10,000 xg, the deproteinized supernatant was collected and stored at –40˚C. The supernatants (20 μL) were analyzed with a JEOL-JLC500/V amino acid analyzer, and amino acids were detected by using ninhydrin reaction (9).

The consumption of each amino acid during incubation was calculated using the following equation:

$$\text{Consumption } (\%) = \{([AA]_{before} - [AA]_{after}) / [AA]_{before}\} \times 100$$

where $[AA]_{before}$ and $[AA]_{after}$ represent the concentration of each amino acid before and after incubation, respectively.

Statistical analysis. Statistical differences among the samples were evaluated using ANOVA followed by Scheffè's multiple comparison test or Student's *t*-test at $\alpha=0.05$.

Results

We found that the consumption of cystine, methionine and histidine was significantly ($p<0.05$) and most prominently enhanced upon contact with Cu plate (Figure 1, Table I), confirming our previous finding (9). It was also noted that the consumption of phenylalanine, arginine, lysine and glutamic acid was also slightly, but not significantly enhanced, whereas the production of glycine was significantly ($p<0.05$) enhanced (Figure 1).

Table I. *Consumption (%) of each amino acid in the culture medium during incubation in the presence or absence of a Cu plate.*

	Control		Saliva		EGCG (0.1 mM)		EGCG (3.0 mM)		Hypoxia	
Amino acid	Cu (–)	Cu (+)	Cu (–)	Cu (+)	Cu (–)	Cu (+)	Cu (–)	Cu (+)	Cu (–)	Cu (+)
Leu	–2.1 (1.8)	–1.0 (1.1)	–9.1 (7.5)	–0.5 (8.0)	–6.0 (5.7)	–8.6 (5.5)	–2.6 (3.0)	–5.8 (4.4)	–5.5 (4.5)	–6.7 (5.4)
IIe	–1.8 (1.1)	–1.9 (0.8)	–3.1 (5.4)	–0.6 (8.5)	–7.1 (6.2)	–9.2 (4.6)	–3.0 (3.4)	–5.4 (4.0)	–4.5 (4.7)	–6.5 (5.2)
Gly	–1.7 (1.8)	–0.9 (1.0)	–67.7 (70.0)	26.9 (32.2)	–6.0 (6.4)	–14.3 (5.5)	0.6 (3.2)	–0.6 (3.8)	23.2 (45.7)	–6.6 (4.5)
Val	–1.6 (1.8)	–1.5 (1.0)	–9.8 (8.8)	–2.0 (5.6)	–5.9 (6.3)	–7.0 (5.0)	–3.0 (3.2)	–1.7 (3.7)	–5.1 (3.7)	–6.1 (4.7)
Cys	**0.0 (1.8)**	**86.7 (3.3)**	**9.8 (3.5)**	**84.7 (2.4)**	**–4.2 (7.9)**	**83.6 (2.7)**	**10.2 (4.1)**	**76.4 (3.5)**	**–3.6 (3.9)**	**23.7 (4.5)**
Ala	–3.4 (2.5)	–6.2 (1.7)	–298.9 (103.4)	–35.6 (25.5)	–7.3 (7.0)	–11.5 (5.6)	–6.0 (3.6)	–4.0 (5.0)	–4.1 (4.7)	–6.0 (4.3)
Met	**–1.1 (3.7)**	**42.6 (1.8)**	**10.8 (7.3)**	**40.4 (2.9)**	**–14.0 (7.2)**	**31.6 (3.2)**	**100.0 (0.0)**	**–146.4 (89.9)**	**–5.4 (4.1)**	**16.4 (3.3)**
Phe	–2.3 (1.6)	2.9 (1.3)	–10.6 (9.8)	2.5 (7.1)	–5.5 (5.8)	–1.4 (5.2)	–1.1 (2.4)	2.0 (4.7)	–4.8 (4.6)	–5.3 (4.8)
Gln	–1.2 (1.6)	–1.4 (0.9)	–2.4 (5.0)	–1.8 (6.6)	–5.5 (6.7)	–6.7 (4.7)	–1.3 (3.4)	–1.7 (4.0)	–4.6 (4.3)	–7.1 (5.0)
Asn	–	–	–	–	–	–	–	–	–	–
Ser	–2.2 (2.0)	–0.3 (0.5)	–11.9 (8.3)	0.5 (8.5)	–5.8 (6.0)	–3.7 (4.8)	–1.6 (3.1)	0.0 (3.7)	–4.5 (4.7)	–6.4 (6.1)
Thr	–1.7 (1.9)	–4.5 (1.1)	–8.6 (6.0)	–5.6 (5.1)	–4.6 (6.7)	–8.1 (5.5)	–6.8 (1.4)	6.1 (2.1)	–4.9 (4.4)	–9.4 (5.2)
Tyr	–1.5 (1.5)	–1.1 (1.4)	–17.5 (5.8)	–1.5 (9.8)	–5.9 (6.5)	–6.4 (5.4)	–1.2 (3.1)	–5.6 (8.2)	–4.9 (4.0)	–5.9 (5.2)
Arg	–0.6 (2.6)	4.9 (0.5)	74.8 (20.8)	5.5 (5.6)	–4.7 (7.5)	0.1 (5.7)	2.0 (2.7)	11.2 (5.5)	–5.2 (4.8)	–6.5 (6.4)
Lys	–2.3 (1.6)	3.2 (2.0)	–8.6 (6.1)	5.2 (5.0)	–5.4 (7.4)	–1.5 (5.2)	3.4 (2.5)	10.8 (4.6)	–5.7 (5.4)	–7.1 (6.4)
His	**–0.9 (1.9)**	**18.8 (1.6)**	**–15.1 (5.3)**	**16.1 (4.0)**	**–5.7 (7.3)**	**15.0 (3.3)**	**4.9 (1.6)**	**10.9 (2.9)**	**–6.0 (5.2)**	**–5.3 (4.3)**
Glu	7.3 (2.2)	22.4 (3.0)	–203.8 (54.8)	18.1 (12.4)	0.8 (3.8)	18.2 (10.8)	4.2 (1.1)	16.4 (0.7)	5.3 (4.0)	13.3 (4.1)

Mean (S.D.)

Plus (+) or minus (–) values represent the consumption and production of amino acid during 30 min, respectively. Each value represents mean±S.D. from three independent experiments.

We investigated the effect of saliva on Cu-induced changes in amino acid consumption. Since the saliva was added at 50% (v/v), the amino acid concentration was corrected for a dilution factor of 2. Addition of saliva to the culture medium without a Cu plate increased the production of glycine, alanine and glutamic acid, and the consumption of arginine. The reason why saliva induced these changes is unexplained. However, the addition of saliva to the medium with a Cu plate did not significantly change the Cu-induced stimulation of cystine, methionine and histidine consumption (Figure 2A, Table I).

We next investigated the effect of EGCG on Cu-induced changes in amino acid consumption. Addition of EGCG to the culture medium induced bi-phasic changes in the cystine, methionine and histidine consumption (Table I). The consumption of these amino acids was reduced at the lower concentration (0.1 mM), possibly by its anti-oxidant action, but was enhanced at higher concentration (3.0 mM), possibly due to its pro-oxidant action. The addition of EGCG to the medium with a Cu plate concentration-dependently reduced the Cu-induced enhancement of cystine, methionine and histidine consumption. In particular, the Cu-induced methionine consumption was completely abrogated at 3.0 mM EGCG (Figure 2B, Table I).

Lastly, we investigated the effect of hypoxia on the Cu-induced changes in amino acid consumption. The consumption of cystine, methionine and histidine was dramatically reduced under hypoxic conditions (Figure 2C). When cells were incubated under hypoxia, the Cu-induced cytotoxicity was significantly reduced, producing higher numbers of viable cells, compared with the cells cultured under normoxic condition (Figure 3).

Discussion

The present study demonstrates that contact with a Cu plate enhanced the consumption of cystine, methionine and histidine, confirming our previous report (9). Since these amino acids are easily oxidizable radical scavengers (11, 12), the enhanced consumption of these amino acids indicates their enhanced oxidation. Among these amino acids, the consumption rate of cystine in the presence of the Cu plate was the greatest, amounting to nearly half of the total amino acid consumption, followed by methionine and histidine. This result is in agreement with our previous finding that the Cu-induced cytotoxicity was almost completely abrogated by *N*-acetyl-L-cysteine addition (9). The enhanced consumption of methionine is coupled to the conversion of methionine into methionine sulfoxide. We have previously observed such conversion of methionine by pro-oxidant action of sodium ascorbate, sodium 5,6-benzylidiene-L-ascorbate, gallic acid or hydrogen peroxide (13). We found that contact with a Cu plate induced a slight increase in the consumption of arginine, suggesting the possible stimulation of nitric oxide production from arginine (14). The increased production of phenylalanine, lysine and

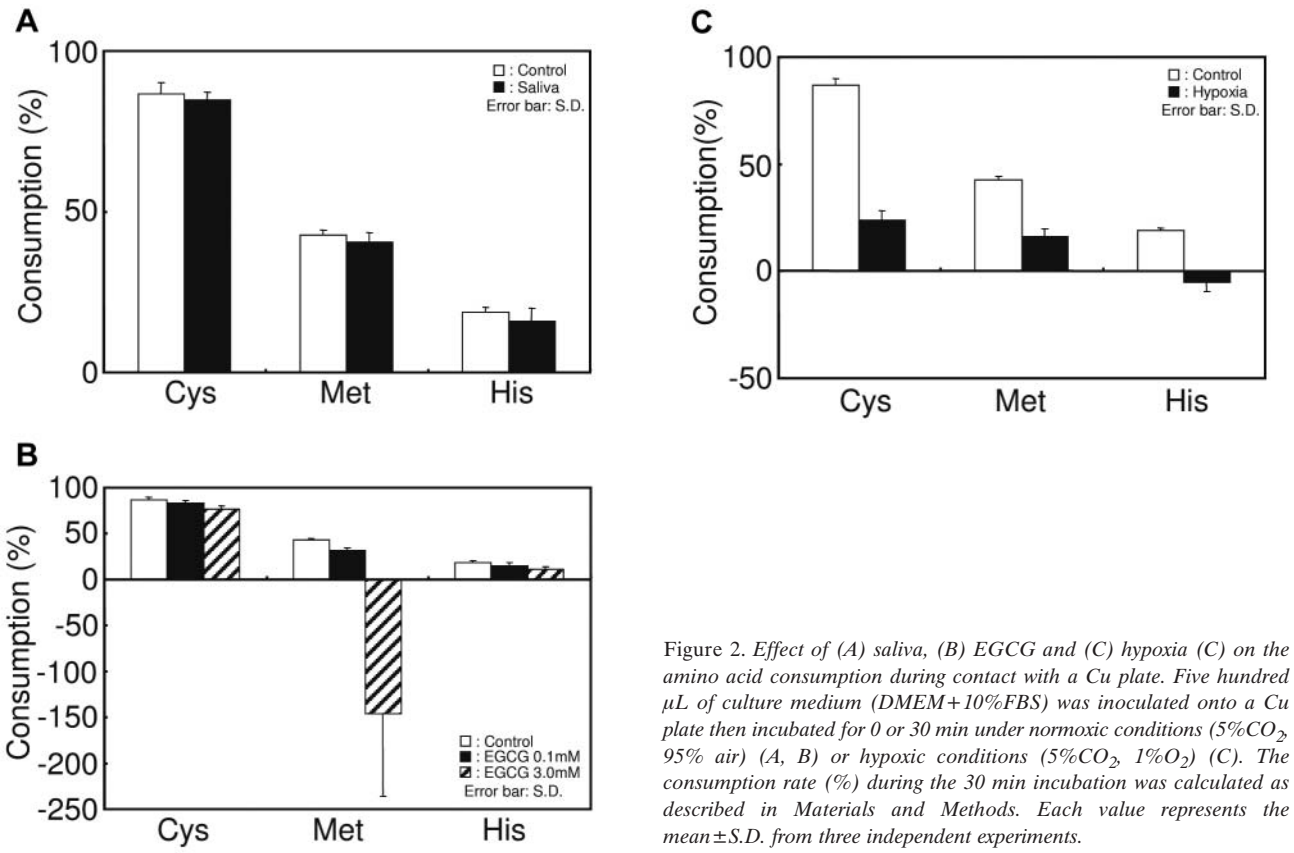

Figure 2. *Effect of (A) saliva, (B) EGCG and (C) hypoxia (C) on the amino acid consumption during contact with a Cu plate. Five hundred μL of culture medium (DMEM+10%FBS) was inoculated onto a Cu plate then incubated for 0 or 30 min under normoxic conditions (5%CO_2, 95% air) (A, B) or hypoxic conditions (5%CO_2, 1%O_2) (C). The consumption rate (%) during the 30 min incubation was calculated as described in Materials and Methods. Each value represents the mean±S.D. from three independent experiments.*

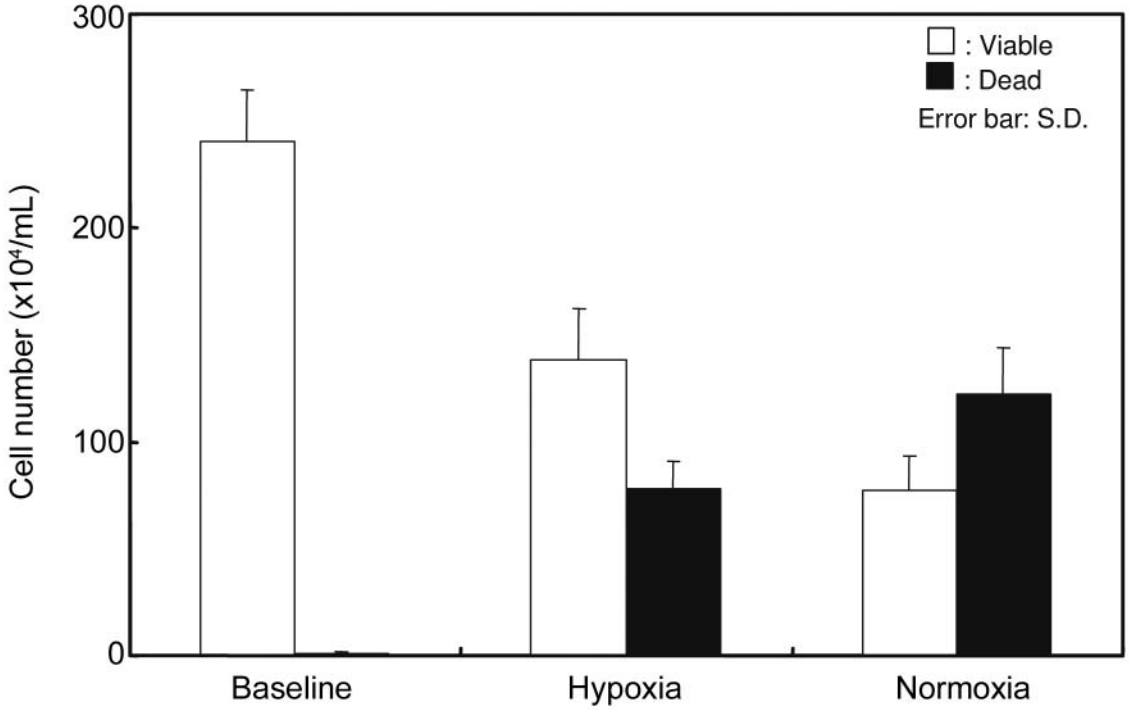

Figure 3. *Effect of hypoxic conditions on the viability of HGF cells during contact with a Cu plate. HGF cells were inoculated onto a Cu plate, incubated for the indicated times under normoxic or hypoxic conditions and the numbers of viable and dead cells were counted using a hemocytometer. Each value represents the mean±S.D. from three independent experiments.*

glutamic acid (Figure 1) is unexplained. The present study also revealed that the contact with the Cu plate induced the production of glycine, an inhibitory transmitter (15). Recently, we found a similar increase in the glycine production when human myelogenous leukemic cell lines (ML-1, KG-1) were committed to die by contact with a silver or palladium plate (17). These data suggest that the enhanced glycine production may contribute, at least in part, to the cytotoxicity induced by contact with metals.

We found that hypoxic conditions most effectively reduced the cytotoxicity and consumption of cystine, methionine and histidine (Figure 2C, Figure 3). This further confirms that Cu-induced cytotoxicity is induced *via* an oxygen-mediated mechanism. However, we could not completely rescue the cells from Cu-induced injury. This may be due to the mild hypoxic conditions (oxygen concentration set to 1%). If we could achieve more rigorous hypoxic conditions, we would expect a higher protective effect. We found that EGCG, in the absence of a Cu plate, had a bi-phasic effect so-called 'hormesis' (16), depending on the concentration: EGCG inhibited the consumption of cysteine, methionine and histidine at a low concentration (0.1 mM), whereas it stimulated the consumption of these amino acids at higher concentration (3 mM) (Table I). However, in the presence of a Cu plate, EGCG brought about only an inhibitory effect. In particular, the prevention of the consumption of methionine, among these three amino acids, by EGCG was the most prominent (Figure 2B, Table I). Since methionine is one of the essential amino acids, such preventive effect of EGCG may profoundly affect the cellular function. The search for more naturally occurring polyphenols should be performed.

Saliva was found to have a very minute effect on the Cu-induced oxidation in the present study (Figure 2A). Since saliva contains various types of cells, bacteria, cytokines, growth factors, amino acids and food remnants, further fractionation of these ingredients may clarify the effect of saliva on the metal-induced oxidative stress.

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