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Copper modulates activities of genistein, nitric oxide, and curcumin in breast tumor cells

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Abstract

Several papers have reported that low level of genistein (<8 μM), the major bioactive component of isoflavones, stimulates the growth of MCF-7 cells. In the present study, we found that genistein-induced growth stimulation of MCF-7 cells is inhibited in the presence of Cu^{2+} (5 μM). Genistein induces the release of nitric oxide in MCF-7 cells in a concentration-dependent manner. The release of nitric oxide was inhibited by N^G-nitro-L-arginine methyl ester, suggesting the possibility of the activation of nitric oxide synthase. The growth of MCF-7 cells also increases in the presence of low levels of sodium nitriprusside (<10 μM), a nitric oxide donor compound, while high levels (>25 μM) are toxic. The sodium nitroprusside-induced growth of MCF-7 cells is drastically suppressed in the presence of Cu^{2+} (5 μM). This parallel behavior between Cu^{2+}–genistein and Cu^{2+}–sodium nitroprusside mixtures suggests that Cu^{2+} and/or copper–protein complexes, that may be formed in the media, may be reacting with nitric oxide or nitric oxide-derived reactive species. The products of these reactions may be responsible for the toxic effects of these mixtures. In contrast, the effect of curcumin that inhibits the growth of both estrogen receptor-positive and -negative breast tumor cells appreciably decreased in the presence of Cu^{2+}. Since copper is known to overwhelmingly bind with proteins, present data suggest that an increase in copper–protein moieties or complexes formed in the serum containing media and their reactions with nitric oxide may be responsible for their toxic effects. Further studies are needed to characterize these reactions.

Keywords: Copper ions; Breast tumor cells; MCF-7 cells; MDA-MB-231 cells; Isoflavones; Genistein; Nitric oxide; Curcumin; Sodium nitroprusside; Cell proliferation

Copper is an essential trace metal and possesses many physiological and pathological activities. Several published studies in cell-free systems have suggested that Cu^{2+} can react with flavones and catechol estrogens producing reactive oxygen species that can oxidatively damage DNA [1,2]. It would be important to determine modulation of the growth enhancing activities of low levels of genistein observed in estrogen responsive breast tumor cells [3,4] by Cu^{2+}.

Genistein, a major bioactive component of isoflavones found in the soybean, has been extensively studied for its anti-tumor and other biological properties [5–7]. In all of these growth inhibitory studies, the concentration of genistein is more than 10 μM, which is not normally achievable in the plasma of humans eating diets even with higher amounts of isoflavones. The maximal attainable peak plasma concentrations of genistein were found to be less than 5 μM [8]. These low levels of genistein have been found to enhance the growth of breast cancer cells in both in vitro and in vivo studies [3,4,9,10].

Nitric oxide (NO) is an important signaling molecule synthesized in the heme pocket of activated nitric oxide synthases (Ca-dependent constitutive cNOS and Ca-independent inducible iNOS) in a two steps process. NO can activate both pro- and anti-proliferative signal transduction pathways in a concentration-dependent manner. In that low levels of NO stimulate growth of tumor cells, while higher levels are toxic to cells. Copper ions can catalyze the liberation of NO from NO-donor compounds such as *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and can rapidly induce oxidation of nitroxyl (NO^-) to nitric oxide [11]. The redox form of

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nitroxyl radical (NO) is formed by several diverse biological reactions. Thionitrites (e.g., nitrosothiols) endogenously occur in various biological systems [12–14] and are thought to be serving as an endogenous reservoir of NO. Glyco-*S*-nitrosothiols were found cytotoxic to DU-145 human prostate cancer cells in vitro [15]. *S*-nitrosothiols and other NO donor compounds have therapeutic potential and their stability and activity can be modulated by copper ions [16].

The anti-inflammatory, anti-oxidant, and anti-tumor growth activities of curcumin, a major component of turmeric, are now well established. Copper ions have been shown to react with curcumin generating DNA-damaging chemicals in cell-free systems [17]. In spite of widely studied chemopreventive activities of curcumin in many types of tumors, it failed to prevent the development of liver and kidney tumors in Long–Evans Cinnamon rats that accumulate copper [18]. Understanding the effect of Cu^{2+} on the bioactivities of curcumin in breast tumor cells will provide important information for dietary and therapeutic purposes.

The objectives of the present studies were to investigate the effects of Cu^{2+} on: (a) the growth enhancing activities of low levels of genistein and that of nitric oxide; and (b) curcumin-induced growth inhibitory activities in breast tumor cells. Our data suggest that Cu^{2+} (5 μM) can inhibit the genistein and NO-induced cell proliferation and that the mixtures of Cu^{2+}–genistein and/or Cu^{2+}–sodium nitroprusside (NO donor molecule) are toxic to breast tumor cells. In contrast, the toxicity of curcumin to breast tumor cells is significantly reduced in the presence of copper ions.

Materials and methods

Breast tumor cells, ER+ (MCF-7 and T47D) and estrogen receptor-negative (ER−) (MDA-MB-231), were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 5 ml of 10,000 U/500 ml medium of penicillin-streptomycin (Gibco-BRL) and insulin in an incubator maintained at 5% CO_2–95% air and 100% humidity at 37 °C. All cells were grown to 80–90%confluence.

Before each experiment with ER+ cells, the media were replaced with phenol red-free RPMI 1640 media supplemented with 10% dextran-coated charcoal treated (DCC) FCS (supplied by Hyclone, Inc. Logan, UT) for 24 h. Media were removed and replaced by a test media containing appropriate concentrations of genistein, NO donor sodium nitroprusside, $CuSO_4$, and curcumin or combinations of these compounds. Control cells were incubated with media containing an equivalent amount of solvent (<0.1%). After 3 days of incubation, the media were replaced with the fresh control and the test media. Cell growth was estimated on day six using tetrazolium assay [3]. Each experiment was performed in triplicate and each data point was repeated at least three times.

For ER− cells (MDA-MB-231), the procedure was exactly as above except that normal RPMI 1640 media plus 10% serum were used instead of DCC supplemented media.

Percent proliferation index (PPI) = 100 × (absorption with test compound/absorption of control) was calculated. The calculated results were analyzed by Student's *t* test. Standard deviation (SD), standard error mean (SEM), and *p* values were determined. Number of cells on day zero were the same in control and in the presence of test compounds.

Measurement of nitric oxide. The amount of nitric oxide (NO) in the supernatant was measured as nitrite plus nitrate, a stable form, using the Griess reagent after reducing nitrate to nitrite. Higher number of cells (1.5×10^6/well) were plated in six well plates in estrogen and dye-free media. Cells were incubated with varying concentrations of genistein at 37 °C. L-Arginine (1 mM) was added to each well. After 24 h, the released NO in the supernatant was measured (as nitrite) spectrophotometrically by using a previously published method [19]. The absorption values were used to calculate the nitric oxide levels from a standard curve constructed between the concentration of sodium nitrite (dissolved in the same media volumes) and absorption at 540 nm (using 38,000 $M^{-1}\,cm^{-1}$ as extinction coefficient) [19].

Growth of MCF-7 cells in the presence of sodium nitroprusside. Sodium nitroprusside (SNP) was used as a NO donor. Stock solution of SNP was made in the media containing DCC-FCS. MCF-7 cells (1.2×10^4) were incubated with varying concentrations of SNP (final concentrations 0–100 μM) at 37 °C. The growth of MCF-7 cells was measured on day six using MTT assay and percent proliferation was calculated as written above. Data were analyzed by Student's *t* test.

Reduction of Cu^{2+} by isoflavones. The reduction of $Cu^{2+} \rightarrow Cu^{+}$ by isoflavones (genistein, diadzein, equol, biochanin A, and formanonetin) was determined spectrophotometrically by using bathocuproinedisulfonic acid (BCS) as the selective Cu^{+} sequestering agent [1]. The stock solutions (10 mM) of BCS and isoflavones were prepared in Tris–HCl buffer (pH 7.4) and in DMSO, respectively. A typical reaction mixture contained 20 μl of isoflavones and 40 μl of Cu^{+} and BCS each in 1 ml of Tris–HCl buffer. The mixture was placed in the dark for 30 min for color development. Absorption was measured at 540 nm using a spectrophotometer.

Results and discussion

Genistein induced cell proliferation and the effect of Cu^{2+} ions

Fig. 1 shows that genistein at low levels stimulates the proliferation of MCF-7 cells confirming the previously published results [3]. Similar data were observed with T47D cells (data not shown). Genistein does not stimulate the growth of ER− MDA-MB-231 cells.

Cu^{2+} ions at concentrations below or at 5 μM do not appreciably influence the growth of MCF-7 or T47D cells. The proliferation of MCF-7 (and of T47D cells, data not shown) decreases when incubated in the mixture of genistein (4 μM) and Cu^{2+} (5 μM) (Fig. 1). Low levels of Cu^{2+} (5 μM) and genistein (4 μM) are not toxic to MDA-MB-231 cells (PPI = 95 ± 5% SEM; SD = 10.1, $p < 0.001$) as has been observed with ER+ cells. However, at higher concentrations of both Cu^{2+} (10 μM) and genistein (>15 μM), the growth of MDA-MB-231 cells is inhibited (PPI = 78 ± 5% SEM; SD = 10.1, $p < 0.001$).

To see the effect of Cu^{2+} ions on genistein pre-loaded cells and vice versa, MCF-7 cells were first exposed to media containing 4 μM of genistein for 24 h followed by washing and adding media containing 5 μM of Cu^{2+}. The proliferation of cells was decreased when measured on day six (PPI = 81 ± 5% SEM; SD = 10.2, $p < 0.001$).

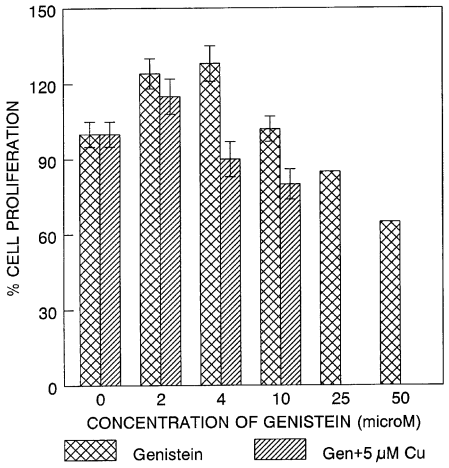

Fig. 1. Effect of genistein concentration without and with the presence of 5 μM Cu^{2+} on the proliferation of MCF-7 cells. Cells (1.5×10^4/well) were exposed to various concentrations of genistein and genistein plus 5 μM $CuSO_4$ mixture. Growth was measured on day six and percent proliferation was calculated as written in the text. Average values from at least three separate experiments ± SEM were used in the plots. SEM = ±5–7%; $p < 0.001$.

This value of PPI is comparable to the value of PPI calculated when both genistein and Cu^{2+} were added together (PPI = 79 ± 5% SEM). In the reverse experiment, that is when cells were first exposed to DCC-FCS media containing 5 μM of Cu^{2+} for 24 h, followed by washing and then incubating with DCC-FCS media containing 4 μM of genistein, cell proliferation on day six (PPI = 127 ± 5% SEM; SD 10.2; $p < 0.001$) is almost similar to that observed with genistein alone without Cu^{2+}.

These results suggest several possibilities. First, Cu^{2+} can oxidize genistein in the media generating oxidized genistein and Cu^{+}. The generation of Cu^{+} was tested spectroscopically in the media without cells. Maximum absorption values at 460 nm of isoflavones–Cu^{2+}–BCS mixture were found in the following order: equol > daidzein > genistein >>> biochanin A. Formanonetin does not reduce Cu^{2+} and also does not stimulate proliferation of MCF-7 cells [3]. The growth stimulation of MCF-7 cells by low levels of isoflavones has been reported in the following order equol = biochanin A > genistein > idzein [3]. Cu^{+} is an unstable ion and the dissolved oxygen can reoxidize it to Cu^{2+}. In order to find out if the growth inhibition is caused by the generation of Cu^{+}, cell proliferation was determined in the presence of media containing bathocuproinedisulfonic acid (BCS), a Cu^{+} sequestering agent [1]. A negligible increase in the percent proliferation of MCF-7 cells under these conditions and a comparison of the order of cell proliferation and Cu-induced oxidation may suggest a minimum involvement of Cu^{+} in growth inhibition of MCF-7 cells. The toxicity of oxidized genistein remained to be tested.

Second, one of the signaling pathways that copper ions can influence may be genistein-induced activation of constitutive nitric oxide synthase (cNOS) and the generation of nitric oxide that is required for cell growth (our unpublished results, paper in preparation). ER+ cells are known to express constitutive nitric oxide synthase (cNOS), while ER− cells do not express this isoform. We have observed that genistein–Cu^{2+} mixture is more toxic to ER+ cells in comparison to the toxicity shown to ER− cells.

We measured genistein-induced release of NO (as nitrite, a stable form) in the supernatant to see if genistein activates cNOS (and/or induces iNOS) in MCF-7 cells. The amount of NO was measured after 24 h of incubation at 37 °C as a function of genistein concentration. Our results show that genistein at 4–8 μM concentrations can generate nitrite in the range of 4.5 μM ± SEM 0.75 μM ($p < 0.01$) that further increases in the presence of 25 and 50 μM genistein (Fig. 2). Genistein-induced (low levels) generation of NO was inhibited by the cNOS inhibitor N^{G}-nitro-L-arginine methyl ester. Genistein-induced (2–8 μM) generation of NO was not observed in experiments performed in Ca–Mg-free PBS or media containing Ca-chelator EGTA. These data suggest that the genistein-induced activation of NOS is calcium-dependent (cNOS). The concentration of nitrite in the supernatant was decreased to the basal level in the presence of 5 μM of Cu^{2+}. This suggests that Cu^{2+} either inhibits the activation of NOS or that Cu^{2+} and/or Cu–protein complexes react with the

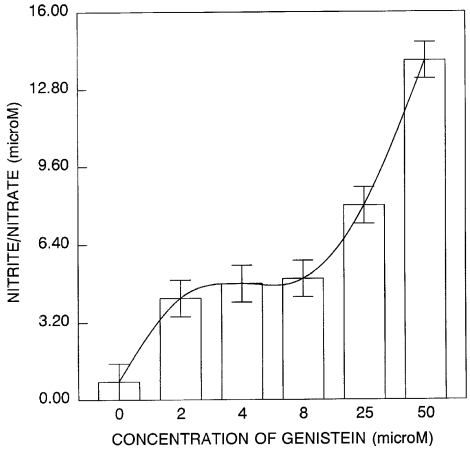

Fig. 2. Effect of genistein concentration on the release of nitric oxide (measured as nitrite) into the supernatant by MCF-7 cells using the Griess reagent. Cells (1.5×10^6/well) were exposed to estrogen-striped media containing genistein (0–50 μM). Nitrite concentration was measured after 24 h of incubation with genistein at 37 °C. The concentrations of nitrite were calculated from the standard curve constructed between concentration of sodium nitrite dissolved in media and absorption at 540 nm (extinction coefficient 38,000 M^{-1} cm^{-1}) following Beer's law. SD ± 1.8; SEM = 0.75 μM). Values are an average of at least three different experiments.

released nitric oxide. Whether genistein can also induce iNOS remains to be tested.

The generated NO and NO-derived products can rapidly react with cellular components like thiols producing *S*-nitrosothiols and other nitrated compounds. Cu^{2+} ions are known to rapidly decompose these NO-donor compounds producing toxic components. This view is partially supported by comparing the proliferation data observed with ER+ and ER− cells. In that, the Cu–genistein mixture is more toxic to MCF-7 and T47D cells in comparison to its toxicity to MDA-MB-231 cells. It appears, therefore, that low levels of Cu^{2+} ions can generate toxicity in the presence of nitric oxide or nitric oxide-derived products. This notion was further supported by our results from experiments with MCF-7 cells exposed to sodium nitroprusside (a NO-donor compound).

Cell proliferation in the presence of sodium nitroprusside (SNP) and effect of Cu^{2+}

Fig. 3 shows the proliferation of MCF-7 cells as a function of the concentration of SNP and SNP plus Cu^{2+} (5 μM). The plot shows SNP-induced biphasic effect, similar to that observed in the presence of genistein (Fig. 1). Cell proliferation in the presence of Cu^{2+} (5 μM) decreases to almost half value to that stimulated by SNP (5 μM). There appears to be a similarity between the growth inhibition pattern shown by the mixtures of SNP–Cu^{2+} and genistein–Cu^{2+}. This parallel behavior may suggest that Cu^{2+} may be reacting with NO or NO-derived reactive species that may be responsible for the cytotoxic effects of genistein–Cu^{2+} or SNP–Cu^{2+} mixture.

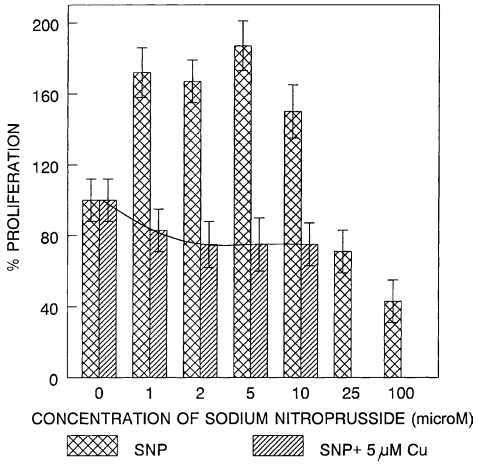

Fig. 3. Effect of sodium nitroprusside (SNP) concentration, without and with the presence of 5 μM of $CuSO_4$ on the proliferation of MCF-7 cells. Stock solution of SNP (10 mM) was prepared in media containing DCC-FCS and appropriate volumes were added to get the desired concentration of SNP. Cell growth was measured on day six. Percent proliferation was calculated as written in the text. Values are an average of at least three different experiments. SEM = ±19 and SD = 39, $p \leqslant 0.001$.

Effect of Cu^{2+} on the curcumin-induced growth inhibition of ER+ and ER− cells

Curcumin has been shown to inhibit the growth of MCF-7 cells, T47D cells, and ER− MDA-MB-231 cells [20]. MDA-MB 231 cells are more sensitive to curcumin in comparison to MCF-7 and T47D cells. The effects of Cu^{2+} on curcumin-induced growth inhibition of MDA-MB-231 and MCF-7 cells are shown in Figs. 4A and B, respectively. Plots show that Cu^{2+} can influence the

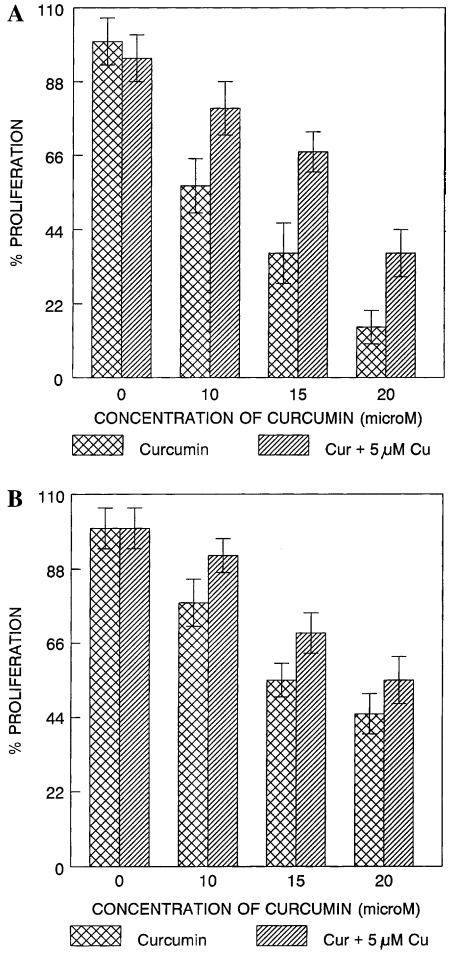

Fig. 4. (A) Effects of curcumin without and with the presence of $CuSO_4$ on the proliferation of MDA-MB-231 cells. Cells (1.5×10^4/well) were incubated with various concentrations of curcumin at 37 °C without and with curcumin plus 5 μM of $CuSO_4$. Growth was measured on day six. Percent proliferation was calculated as written in the text. Values are an average of at least three separate experiments. SEM = ±5–7%; $p < 0.001$. (B) Effects of curcumin without and with the presence of $CuSO_4$ on the proliferation of MCF-7 cells. Cells (1.5×10^4/well) were incubated at 37 °C with various concentrations of curcumin and with curcumin plus 5 μM of $CuSO_4$. Percent proliferation was calculated as written in the text. Values are an average of at least three different experiments. SEM = ±5–7%.

growth-inhibitory effects of curcumin. This effect of Cu^{2+} is more pronounced in MDA-MB-231 cells than that observed in MCF-7 (Figs. 4A and B). These data are in contrast to the conclusion derived from cell-free systems where curcumin–copper ion mixture produced DNA damaging species [17]. We studied spectroscopically the reaction between curcumin and Cu^{2+} ions in the dye-free tissue culture media containing 5% serum but without cells. No shift or broadening of the maximum absorption peak of curcumin was observed in the presence of Cu^{2+} as has been reported in buffers without serum and media chemicals [17]. This may suggest that Cu^{2+} ions in the presence of media proteins may not be reacting with curcumin to generate DNA damaging species [17]. Copper is known to overwhelmingly bind with proteins. It is likely that the effects seen here may be in part due to an increase in copper–protein moieties or complexes formed in the serum containing media, which influences the activity of native curcumin.

Studies are in progress to determine genistein-induced activation/expression of nitric oxide synthases (cNOS and/or iNOS) and characterization of reaction products of copper with nitric oxide and serum protein(s).

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