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Review

GHK-Cu may Prevent Oxidative Stress in Skin by Regulating Copper and Modifying Expression of Numerous Antioxidant Genes

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Abstract: The copper binding tripeptide GHK (glycyl-L-histidyl-L-lysine) is a naturally occurring plasma peptide that significantly declines during human aging. It has been established that GHK:Copper(2+) improves wound healing and tissue regeneration and stimulates collagen and decorin production. GHK-Cu also supports angiogenesis and nerve outgrowth, improves the condition of aging skin and hair, and possesses antioxidant and anti-inflammatory effects. In addition, it increases cellular stemness and secretion of trophic factors by mesenchymal stem cells. GHK's antioxidant actions have been demonstrated *in vitro* and in animal studies. They include blocking the formation of reactive oxygen and carbonyl species, detoxifying toxic products of lipid peroxidation such as acrolein, protecting keratinocytes from lethal Ultraviolet B (UVB) radiation, and blocking hepatic damage by dichloromethane radicals. In recent studies, GHK has been found to switch gene expression from a diseased state to a healthier state for certain cancers and for chronic obstructive pulmonary disease (COPD). The Broad Institute's Connectivity Map indicated that GHK induces a 50% or greater change of expression in 31.2% of human genes. This paper reviews biological data demonstrating positive effects of GHK in skin and proposes interaction with antioxidant-related genes as a possible explanation of its antioxidant activity.

Keywords: copper peptides; GHK; glycyl-L-histidyl-L-lysine; reactive oxygen species; reactive carbonyl species; ferritin iron oxidation; dichloromethane; gene profiling; gene expression

1. Introduction

Human skin serves as a barrier between the internal environment of the body and the external environment. As such, it is constantly exposed to a multitude of potentially damaging factors. One of the most important environmental factors is ultraviolet (UV) radiation, which can lead to the generation of potentially damaging reactive oxygen species (ROS). Today, ROS are implicated in skin aging, cancer, and pigmentation disorders [1].

Robust protective systems and intricate biochemical pathways ensure that skin remains healthy and that any damage that occurs is repaired in a timely manner. However, there are many situations, such as excessive sun tanning, in which these systems become overwhelmed and cannot cope with environmental challenges. Some of the resulting damage is short-lived and can be easily repaired; however, some can have serious consequences, such as skin cancer [2].

Recently, it was established that many naturally occurring compounds known for their ability to prevent oxidative stress and inflammation are capable of regulating multiple biochemical pathways by up- or down-regulating gene expression [3]. This opened a new venue for skin aging and cancer prevention research.

This article discusses the naturally occurring tripeptide copper complex GHK-Cu, which is known to possess wound healing, antioxidant, anti-inflammatory, and anti-aging properties [4]. Recent gene profiling studies demonstrated its ability to regulate the expression of a number of human genes. Even though there is still not enough data to connect gene profiling data to biological effects, we identified some interesting parallels, which may suggest a possible mechanism of GHK-Cu actions. [5]. Here we will focus primarily on antioxidant properties of the GHK-Cu peptide.

2. Chemistry of the GHK-Cu Complex

The tripeptide GHK was isolated in 1973 from human plasma albumin by Loren Pickart. It was identified as an activity that markedly stimulated CO_2 production and lipid synthesis in liver slices and stimulated protein, RNA, and DNA production in neoplastic and normal liver cell culture. It also increased cell growth in hepatoma cells and promoted survival in normal liver cells. Comparison of the purified tripeptide to the synthetic peptides and later structural analysis established its amino acid sequence as glycyl-L-histidyl-L-lysine [6–8]. GHK is naturally present in the body as a part of collagen molecules and is released from collagen after an injury. Also, experiments by Sage *et al.* revealed that a protein named SPARC releases GHK after tissue breakdown. SPARC is primarily expressed during embryonic development or in the course of tissue healing and remodeling [9].

Since the structure of the GHK peptide resembled the copper chelating sites of proteins, albumin in particular, Pickart suggested that the tripeptide may act by chelating an important metal factor and delivering it to the cells. Consequent studies confirmed that the tripeptide had a strong affinity for

copper, readily forming the complex GHK-Cu [10]. In human plasma GHK is present at about 200 μg/L in men of age 20–25, but declines to 80 μg/L by age 60–80. See Figure 1.

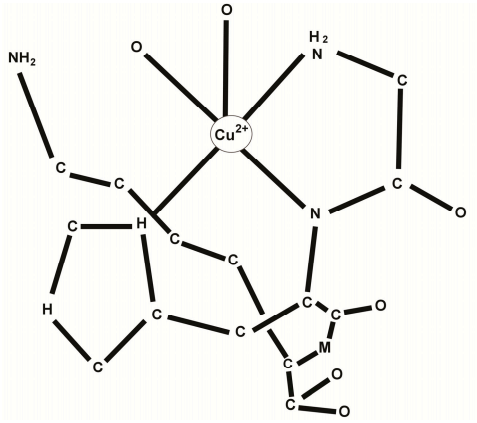

Figure 1. Molecular structure of the tri-peptide GHK-Cu.

Due to its high affinity to copper 2+, GHK can obtain copper ions bound to other molecules such as albumin. Since albumin is the main copper-transporting molecule in human plasma, GHK's ability to obtain copper ions from albumin enables it to serve as a delivery vehicle for copper ions locally, for example, in a site of an injury [11].

3. Biology of the GHK-Cu Complex

One of the earliest observed effects of the GHK peptide was its ability to stimulate growth of cultured cells [12]. GHK promoted growth of KB and HeLa human cells when used in serum limited medium with bovine serum albumin as compared to the media containing 5% serum. The synthetic peptides used as the control produced no effect or slowed down cell growth [13]. When GHK was added to cells that were forced into dormancy by serum deprivation, they would re-enter the cell cycle [14].

A recent study showed the ability of GHK-Cu to restore function of irradiated fibroblasts to that of intact cells. GHK-Cu-treated irradiated fibroblasts showed much faster growth compared to the normal (non-irradiated) control cells. In addition, irradiated fibroblasts treated with GHK-Cu produced significantly more basic fibroblast growth factor (bFGF) and vascular endothelium growth factor (VGF) compared to both irradiated and intact control cells. The production of transforming growth factor beta (TGF-beta) was not affected [15].

4. GHK as a Gene Expression Modifier

Iorio *et al.* used a repository of transcriptional responses to compounds, the Connectivity Map (cMap) [16], and MANTRA software (www.mantra.tigem.it) to explore networks of compounds producing similar transcriptional responses. GHK, as one of the compounds studied, increased mRNA production in 268 genes while suppressing 167 [17].

In another study that used the cMap, GHK was identified as one of two perturbagens that could significantly reverse the expression of 70% of 54 human genes overexpressed in patients with an aggressive metastatic form of colon cancer. Another compound was a plant alkaloid, securinine. Since

both compounds were identified among a host of 1309 biologically active compounds and were active at a very low non-toxic concentration (1 μM for GHK and 18 μM for securinine), the authors suggested them as potential candidates for adjuvant chemotherapy [18].

A recent impressive collaborative study conducted by scientists from Boston University, University of Groningen (The Netherlands), University of British Columbia, and University of Pennsylvania established that the GHK peptide reverses the gene expression signature of emphysematous lung destruction. It was established that there were 127 genes associated with the severity of emphysema. For example, genes linked to increased inflammation displayed increased activity, while genes linked to repair displayed decreased activity.

Using the cMap, it was demonstrated that with addition of GHK the expression of pro-inflammatory genes in COPD lung tissue was reduced and the expression of repair genes was restored. These changes in gene expression corresponded to changes in fibroblasts' function observed *in vivo*. Furthermore, when lung fibroblasts from emphysema lungs were incubated with GHK peptide, they showed great improvement in their ability to remodel collagen and assemble it into fibrils [19].

Our own query of the cMap yielded a substantial number of human genes that were up- or down-regulated by GHK. We are well aware that at the current level of knowledge, there is not enough information that would allow us to establish a clear relationship between changes in gene expression, obtained through gene profiling, and an actual biological activity observed in experiments. However, we find it intriguing that among genes whose expression is affected by GHK, there is a large number of genes that play important roles in the aging process [20]. These findings are consistent with a large body of experimental evidence that confirms broad-range anti-aging and health supporting activity of the GHK-Cu. In most experiments the GHK-Cu complex was used, which seems to be the most active form, but few studies were performed with the GHK peptide without copper. However, since the GHK peptide has such a high affinity for copper, it is virtually impossible to ensure that no GHK-Cu was present, and we will mostly focus on the effects of its copper complex.

5. Wound Healing and Tissue Remodeling Activity of GHK-Cu

Pickart *et al.* were first to establish that GHK-Cu accelerated wound healing and contraction, improved the take of transplanted skin, and also possessed anti-inflammatory actions [21,22].

Subsequent studies directed by Borel and Maquart *et al.* demonstrated that both GHK and GHK-Cu, at a very low, non-toxic concentration (0.01–1 nM), stimulated collagen synthesis in cultured fibroblasts while non-collagen proteins were not affected [23]. In another study, the same group established that GHK-Cu increased collagen I and collagen III expression when injected in experimental wounds in rats. The increase was detected in the samples collected on day 3 and persisted until day 14. The expression of TGF-beta was not changed [24]. GHK-Cu also stimulated both the synthesis and breakdown of collagen and glycosaminoglycans [25]. It increased expression of both metalloproteinases and their inhibitors, acting as a main regulator of wound healing and skin remodeling processes. Since only GHK-Cu, but not GHK, exhibited those properties, it was concluded that the copper-binding activity of GHK is essential for its wound healing and skin remodeling effects [26].

In 2000, the same group demonstrated, using mRNA analysis, that GHK-Cu stimulated the production of collagen, dermatan sulfate, chondroitin sulfate, and the small proteoglycan decorin [27].

GHK-Cu also accelerated wound healing in animal experiments by improving circulation, increasing activity of antioxidant enzymes, and encouraging epithelization [28–33].

6. Antioxidant Activity of GHK-Cu

The antioxidant actions of GHK have been demonstrated *in vitro* and in animal wound healing studies. They include inhibiting the formation of reactive carbonyl species (RCS), detoxifying toxic products of lipid peroxidation such as acrolein, protecting keratinocytes from lethal UVB radiation, and preventing hepatic damage by dichloromethane radicals.

The ability of GHK to prevent oxidative stress was tested in vitro using Cu(2+)-dependent oxidation of low-density lipoproteins (LDL). LDL were treated with 5 μM Cu(2+) for 18 h in either phosphate buffered saline (PBS) or Ham's F-10 medium. There was increased production of thiobarbituric acid reactive substances (TBARSs), which indicated increased oxidation. GHK and histidine "entirely blocked" (author's words) the *in vitro* Cu(2+)-dependent oxidation of low-density lipoproteins (LDL). In comparison, superoxide dismutase (SOD1) provided only 20% reduction of oxidation [34].

Acrolein, a well-known carbonyl toxin, is produced by lipid peroxidation of polyunsaturated fatty acids. GHK effectively reduces the formation of both acrolein and another product of oxidation, 4-hydroxynonenal [35,36].

GHK also blocks lethal ultraviolet radiation damage to cultured skin keratinocytes by binding and inactivating reactive carbonyl species such as 4-hydroxynoneal, acrolein, malondialdehyde, and glyoxal [37].

The intraperitoneal injection of 1.5 mg/kg of GHK into rats for five days before dichloromethane poisoning and five days thereafter provided protection of the functional activity of hepatocytes and immunological responsiveness. Dichloromethane is toxic to hepatic tissue via the formation of a dichloromethane free radical that induces acute toxic damage [38].

In rats with experimental bone fractures peptides, GHK (0.5 μg/kg), dalargin (1.2 μg/kg), and thymogen (0.5 μg/kg) were injected intraperitoneally. Within 10 days there was a decrease of malonic dialdehyde and an increase of catalase activity in blood. There was also a marked increase of reparative activity. Each combination of peptides was more potent than any of the studied peptides injected separately. The synergetic action of peptides Gly-His-Lys, thymogen, and dalargin was proposed for stimulation of reparative osteogenesis [39].

GHK-Cu reduced iron release from ferritin by 87%. Iron has also been shown to have a direct role in the initiation of lipid peroxidation. An Fe(2+)/Fe(3+) complex can serve as an initiator of lipid oxidation. In addition, many iron complexes can catalyze the decomposition of lipid hydroperoxides to the corresponding lipid alkoxy radicals. The major storage site for iron in serum and tissue is ferritin. Ferritin in blood plasma can store up to 4500 atoms of iron per protein molecule and superoxide anion can promote the mobilization of iron from ferritin. This free iron may then catalyze lipid peroxidation and the conversion of superoxide anion to the more damaging hydroxyl radical [40].

6.1. Synthesis of GHK-Cu Analogs with Higher Anti-ROS Activity

GHK-Cu has, on a molar basis, about 1% to 3% of the activity of the Cu, Zn superoxide dismutase protein. By simple modifications to the peptide, it is possible to raise the SOD-mimetic activity up 223-fold [41]. Given the broad range of the antioxidant actions of GHK, it is likely that modifications

that increase SOD-mimetic activity would also change, possibly increasing their suppression of other reactive species such as RCS and dichloromethane radicals. See Table 1.

Table 1. SOD-mimetic activity of GHK-Cu chemical analogs.

Chemical Structure	SOD-Mimetic Activity Compared to GHK:Cu(2+)
GHK:Cu(2+)	100 (base line)
KHG-Amide:Cu(2+)	21
GHKAFA:Cu(2+)	561
AHK:Cu(2+)	563
GHK-Octyl Ester:Cu(2+)	810
GHCaprolactam:Cu(2+)	4500
HGK:Cu(2+)	22,300

6.2. Antioxidant Gene Expression Analysis

Our group used Broad Institute's cMap to acquire gene expression data (retrieved 5 March 2013). The cMap is a large database that contains more than 7000 gene expression profiles of five human cell lines treated with 1309 distinct small molecules. Three GHK profiles are contained in this repository. These profiles were created using the GeneChip HT Human Genome U133A Array. Among the five cell lines used by the cMap, only two were treated with GHK. Two of the profiles were created using the PC3 cell line while the third used the MCF7 cell line. Our studies utilized all three gene expression profiles.

In order to analyze the gene data obtained from the cMap, we used GenePattern. GenePattern is a publicly available computational biology open-source software package developed at the Broad Institute of MIT and Harvard for the analysis of genomic data. The CEL files were processed with Microarray Analysis Suite 5 (MAS5) and background correction. Files were then uploaded to the ComparativeMarkerSelectionViewer module in order to view fold changes for each probe set.

Due to multiple probe sets mapping to the same gene, we converted the fold changes in m-RNA production produced by GenePattern to percentages, then averaged all probe sets representing the same gene. It was determined that the 22,277 probe sets in the Broad Institute's data represent 13,424 genes. This ratio (1.66) was used to calculate the overall number of genes that affect GHK at various cutoff points (rather than probe sets) [42].

Table 2 is an estimate of the number of genes affected by GHK at various cutoff points (number of probe sets mapping to the same gene divided by 1.66). The percentage of genes stimulated or suppressed by GHK with a change greater than or equal to 50% is 31.2%.

Table 2. Estimate of number of genes affected by GHK.

Percent Change	Genes Stimulated	Genes Suppressed
50%–99%	1569	583
100%–199%	646	469
200%–299	227	196
300%–599%	196	207
600%–899%	39	42
900%–1199%	8	7
1200% or more	2	4

A search of antioxidant associated genes effected by GHK yielded 17 genes with significant antioxidant activity. See Table 3.

Table 3. Gene expression related to antioxidant activity.

Genes	Percent Change in Gene Expression *	Comments
TLE1	762	Inhibits the oxidative/inflammatory gene NF-κB [43].
SPRR2C	721	This proline-rich, antioxidant protein protects outer skin cells from oxidative damage from ROS. When the ROS level is low, the protein remains in the outer cell membrane, but when the ROS level is high, the protein clusters around the cell's DNA to protect it [44,45].
ITGB4	609	Up-regulation of ITGB4 promotes wound repair ability and antioxidative ability [46].
APOM	403	Binds oxidized phospholipids and increases the antioxidant effect of high-density lipoproteins (HDL) [47].
PON3	319	Absence of PON3 (paraoxonase 3) in mice resulted in increased rates of early fetal and neonatal death. Knockdown of PON3 in human cells reduced cell proliferation and total antioxidant capacity [48].
IL18BP	295	The protein encoded by this gene is an inhibitor of the pro-inflammatory cytokine IL18. IL18BP abolished IL18 induction of interferon-gamma (IFNgamma), IL8, and activation of NF-κB *in vitro*. Blocks neutrophil oxidase activity [49].
HEPH	217	Inhibits the conversion of Fe(2+) to Fe(3+). HEPH increases iron efflux, lowers cellular iron levels, suppresses reactive oxygen species production, and restores mitochondrial transmembrane potential [50].
GPSM3	193	Acts as a direct negative regulator of NLRP3. NLRP3 triggers the maturation of the pro-inflammatory cytokines IL-1β and IL-18 [51].
FABP1	186	Reduces intracellular ROS level. Plays a significant role in reduction of oxidative stress [52,53].
PON1	149	PON1 (paraoxonase 1) is a potent antioxidant and a major anti-atherosclerotic component of HDL [54].
MT3	142	Metallothioneins (MTs) display *in vitro* free radical scavenging capacity, suggesting that they may specifically neutralize hydroxyl radicals. Metallothioneins and metallothionein-like proteins isolated from mouse brain act as neuroprotective agents by scavenging superoxide radicals [55,56].
PTGS2	120	Produces cyclooxygenase-II (COX-II), which has antioxidant activities [57].
SLC2A9	117	The p53-SLC2A9 pathway is a novel antioxidant mechanism. During oxidative stress, SLC2A9 undergoes p53-dependent induction, and functions as an antioxidant by suppressing ROS, DNA damage, and cell death [58].
NFE2L2	56	Nuclear respiratory factor 2 helps activate antioxidant responsive element-regulated genes which contribute to the regulation of the cellular antioxidant defense systems [59].
PTGS1	50	Produces cyclooxygenase-I (COX-I), which has antioxidant activity [57].
TNF	−115	GHK suppresses this pro-oxidant TNF gene [60].
IL17A	−1018	This cytokine can stimulate the expression of IL6 and cyclooxygenase-2 (PTGS2/COX-2), as well as enhance the production of nitric oxide (NO). High levels of this cytokine are associated with several chronic inflammatory diseases including rheumatoid arthritis, psoriasis, and multiple sclerosis (http://www.ncbi.nlm.nih.gov/gene/).

***** Probe sets were averaged when multiple probes mapped to the same gene. Only probe sets with changes equal to or greater than 50% were included.

7. Conclusions

The GHK peptide is currently a popular ingredient in skin care products [60]. However, the exact mechanism of its actions remains to be established. Currently, all we can say is that both copper-transporting functions of GHK-Cu and its ability to modify gene expression have biological significance.

Animal and *in vitro* studies present overwhelming evidence of the widespread positive effects of GHK in the human body. Until recently it was not quite clear how one simple molecule can possess so many biological effects ranging from the stimulation of wound healing and tissue regeneration to the increase in secretion of important biological molecules and antioxidant defense. Based on the current gene profiling data, we can hypothesize that the diverse effects of GHK can occur at least partially through modifying gene expression. It is possible that GHK can improve the skin's antioxidant defense not only by supporting SOD activity, quenching toxic products of lipid peroxidation, and modulating the iron level, but also by modifying expression of multiple antioxidant-related genes. It is important to keep in mind that even though today there are many compounds that display impressive gene-regulating activity in computer-based gene profiling studies, not all of them have supporting biological studies. Future research is needed to conduct more detailed transcriptional analyses, interpret the gene data, and establish its connection to biological data.

GHK is readily available at low cost and can be easily incorporated into a wide range of skin products such as sunscreens and protective cosmetic creams, as well as medicated ointments. It penetrates the *stratum corneum* and can be incorporated into liposomes or skin patches [61–64]. The molecule is very safe and no issues have ever arisen during its use as a skin cosmetic or in human wound healing studies.

Author Contributions

The authors have equally contributed for writing and revision of this article.

Conflicts of Interest

The authors declare no conflict of interest.

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