Reduction in the E2k Subunit of the α-Ketoglutarate Dehydrogenase Complex Has Effects Independent of Complex Activity*

Received for publication, August 9, 2004, and in revised form, January 12, 2005 Published, JBC Papers in Press, January 12, 2005, DOI 10.1074/jbc.M409064200

Qingli Shi, Huan-Lian Chen, Hui Xu, and Gary E. Gibson‡

From the Department of Neurology and Neuroscience, Weill Medical College of Cornell University at Burke Medical Research Institute, White Plains, New York 10605

The activity of the α-ketoglutarate dehydrogenase complex (KGDHC) declines in brains of patients with several neurodegenerative diseases. KGDHC consists of multiple copies of E1k, E2k, and E3. E1k and E2k are unique to KGDHC and may have functions independent of the complex. The present study tested the consequences of different levels of diminished E2k mRNA on protein levels of the subunits, KGDHC activity, and physiological responses. Human embryonic kidney cells were stably transfected with an E2k sense or antisense expression vector. Sense control (E2k-mRNA-100) was compared with two clones in which the mRNA was reduced to 67% of control (E2k-mRNA-67) or to 30% of control (E2k-mRNA-30). The levels of the E2k protein in clones paralleled the reduction in mRNA, and E3 proteins were unaltered. Unexpectedly, the clone with the greatest reduction in E2k protein (E2k-mRNA-30) had a 40% increase in E1k protein. The activity of the complex was only 52% of normal in E2k-mRNA-67 clone, but was near normal (90%) in E2k-mRNA-30 clone. Subsequent experiments tested whether the physiological consequences of a reduction in E2k mRNA correlated more closely to E2k protein or to KGDHC activity. Growth rate, increased DCF-detectable reactive oxygen species, and cell death in response to added oxidant were proportional to E2k proteins, but not complex activity. These results were not predicted because subunits unique to KGDHC have never been manipulated in mammalian cells. These results suggest that in addition to its essential role in metabolism, the E2k component of KGDHC may have other novel roles.

Received, October 3, 2004, and in final form, January 12, 2005

Published, JBC Papers in Press, January 12, 2005, DOI 10.1074/jbc.M409064200

Qingli Shi, Huan-Lian Chen, Hui Xu, and Gary E. Gibson‡

From the Department of Neurology and Neuroscience, Weill Medical College of Cornell University at Burke Medical Research Institute, White Plains, New York 10605

The activity of the α-ketoglutarate dehydrogenase complex (KGDHC) declines in brains of patients with several neurodegenerative diseases. KGDHC consists of multiple copies of E1k, E2k, and E3. E1k and E2k are unique to KGDHC and may have functions independent of the complex. The present study tested the consequences of different levels of diminished E2k mRNA on protein levels of the subunits, KGDHC activity, and physiological responses. Human embryonic kidney cells were stably transfected with an E2k sense or antisense expression vector. Sense control (E2k-mRNA-100) was compared with two clones in which the mRNA was reduced to 67% of control (E2k-mRNA-67) or to 30% of control (E2k-mRNA-30). The levels of the E2k protein in clones paralleled the reduction in mRNA, and E3 proteins were unaltered. Unexpectedly, the clone with the greatest reduction in E2k protein (E2k-mRNA-30) had a 40% increase in E1k protein. The activity of the complex was only 52% of normal in E2k-mRNA-67 clone, but was near normal (90%) in E2k-mRNA-30 clone. Subsequent experiments tested whether the physiological consequences of a reduction in E2k mRNA correlated more closely to E2k protein or to KGDHC activity. Growth rate, increased DCF-detectable reactive oxygen species, and cell death in response to added oxidant were proportional to E2k proteins, but not complex activity. These results were not predicted because subunits unique to KGDHC have never been manipulated in mammalian cells. These results suggest that in addition to its essential role in metabolism, the E2k component of KGDHC may have other novel roles.

Diminished α-ketoglutarate dehydrogenase complex (KGDHC) activities have been consistently observed in both normal and pathologically involved brain areas of patients with Alzheimer’s disease (AD) (1–4), Parkinson’s disease (5–8), progressive supranuclear palsy (9–10), and Wernicke-Korsakoff syndrome (11). The reduction in KGDHC activities in brains from AD patients bearing one apolipoprotein E4 allele is highly correlated to a clinical dementia rating (12). Reduced KGDHC activity is also found in fibroblasts from some AD patients (13). These results suggest that the change in KGDHC activity is not merely a secondary event of neurodegeneration (14–15), and that multiple factors may act on KGDHC to make it a point of convergence in these disorders. The current experiments test the consequences of a reduction of one component of KGDHC.

KGDHC is a key and arguably rate-controlling enzyme of the tricarboxylic acid cycle. KGDHC consists of three subunits: α-ketoglutarate dehydrogenase (E1k, EC 1.2.4.2), dihydrolipoyl succinyltransferase (E2k, EC 2.3.1.61), and dihydrolipoyl dehydrogenase (E3, EC 1.8.1.4). E1k and E2k are unique to KGDHC whereas E3 is also a component of the pyruvate dehydrogenase complex (PDHC) and branched-chain α-ketoadipate dehydrogenase complex (BCH). Structural studies of the Escherichia coli KGDHC demonstrate that 6 dimers of E1k and 6 dimers of E3 are arranged along an octahedral E2k core, which contains 24 polypeptide chains and only half of these chains have covalently bound lipoic acid (16). Although the interactions of these subunits have been studied extensively in yeast and bacteria, previous studies have not been performed in mammalian cells.

Oxidative stress is one plausible link between KGDHC deficiency and neurodegeneration. Accumulating evidence indicates that oxidative stress occurs in brains of patients with AD (17–18) and other neurodegenerative diseases (9, 19–23). Oxidative stress can also lead to cell death (24). For example, exposure of Hela or CHO cells to 80% O2 for 2 days inhibits cell growth and leads to loss of reproductive capacity (25–26). In CHO cells, loss of cell growth correlates with total inhibition of KGDHC (26). H2O2 also reduces KGDHC activity in intact mitochondrial (27), synaptosomes (28), fibroblasts (29) and N2a cells (30). However, the mechanisms that link diminished KGDHC activity, oxidative stress, and cell death are unclear.

Each of the KGDHC subunits has unique enzymatic functions. Their response to H2O2 and roles in cell death may also differ. Whether the protein levels (i.e. immunoreactivity) of the three subunits of KGDHC decline with disease is variable in both genetic and sporadic forms of the AD (15, 31). The three subunits also show a selective response to oxidative stress (32). Thus, to understand the response of KGDHC to disease and to oxidative stress, it is essential to investigate the roles of each subunit in normal biological function and in the cellular response to oxidative stress. The results will help us to elucidate the mechanism of the diminished KGDHC activity in neurodegenerative diseases.

E2k, as a component of KGDH complex core, plays an essential role in mediating E1k-catalyzed decarboxylation of α-keto-
glutarate and reductive acylation of the lipoic moiety and E3-catalyzed reoxidation of the dihydrodiol moiety (33). For example, radicals generated by E2k can inhibit E1k (34). The interaction of KGDH with the reactive oxygen species (ROS)-dependent pathways provides for novel function(s) of the complex. For instance, in mycobacterium tuberculosis (Mtcb), the KGDH-bound dihydrolipoate intermediate, a thioredoxin-like protein and a thioredoxin-dependent peroxidase comprise the specific antioxidant defense system (35).

Thus, E2k was genetically manipulated to different levels in human embryonic kidney cells (HEK293) using an antisense RNA strategy. The consequences of reducing E2k mRNA by about one-third and two-thirds on cellular function under normal conditions and following oxidative stress were analyzed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The parental cell line of Flp-In-293 was a human embryonic kidney cell line (HEK293). Flp-In-293 cells (Invitrogen, Carlsbad, CA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and zeocin (100 μg/ml), and incubated at 37 °C in a humidified atmosphere of 5% CO2 in air. Flp-In-293 cell lines expressing sense or antisense RNA of E2k were grown in the same medium without zeocin although with the addition of hygromycin (100 μg/ml).

**Cloning and Construction of E2k Sense and Antisense Expression Vectors**—An antisense RNA strategy was used to manipulate the expression of E2k. BLAST searching was performed to ensure no homology between the designed E2k antisense DNA and other off-target genes. In brief, Flp-In-293 cells were grown in a 6-well plate. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the supplier's instructions. This was followed by first strand cDNA synthesis using the First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche). The antisense RNA was synthesized using the First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche). This was followed by first strand cDNA synthesis using the First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche). Using the above procedure, an antisense RNA strategy was used to manipulate the expression of E2k.

**Generation of Stable E2k Sense and Antisense Expression Vectors**—Antisense RNA strategy was used to manipulate the expression of E2k. BLAST searching was performed to ensure no homologies between the designed E2k antisense DNA and other off-target genes. In brief, Flp-In-293 cells were grown in a 6-well plate. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the supplier's instructions. This was followed by first strand cDNA synthesis using the First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche). The antisense RNA was synthesized using the First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche). Using the above procedure, an antisense RNA strategy was used to manipulate the expression of E2k. After incubation of cells at 37 °C in a humidified atmosphere of 5% CO2 in air, Flp-In-293 cell lines expressing sense or antisense RNA of E2k were grown in the same medium without zeocin although with the addition of hygromycin (100 μg/ml).

**Cloning and Construction of E2k Sense and Antisense Expression Vectors**—An antisense RNA strategy was used to manipulate the expression of E2k. BLAST searching was performed to ensure no homologies between the designed E2k antisense DNA and other off-target genes. In brief, Flp-In-293 cells were grown in a 6-well plate. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the supplier's instructions. This was followed by first strand cDNA synthesis using the First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche). The antisense RNA was synthesized using the First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche). Using the above procedure, an antisense RNA strategy was used to manipulate the expression of E2k. After incubation of cells at 37 °C in a humidified atmosphere of 5% CO2 in air, Flp-In-293 cell lines expressing sense or antisense RNA of E2k were grown in the same medium without zeocin although with the addition of hygromycin (100 μg/ml).

**KGDHC Activity Assays**—KGDHC activities were also determined with an in situ histochemistry assay (36) with some modifications. The basis of the assay was to distinguish NADH production from KGDHC to that from other enzymes (i.e. substrate specificity). One method assays activity in cells whereas the other assesses the activity in slightly permeabilized cells in which the mitochondria are nearly intact (in situ assay).

**KGDH Activity Assays**—Two independent, well-established assays were used to measure KGDH activities. KGDH catalyzes the following reaction: α-ketoglutarate + NAD+ + CoA → succinyl CoA + CO2 + NADH. Both methods use the specific substrate α-ketoglutarate (α-KG) to distinguish NADH production from KGDH to that from other enzymes (i.e. substrate specificity). One method assays activity in cells whereas the other assesses the activity in slightly permeabilized cells in which the mitochondria are nearly intact (in situ assay).

**KGDH Activity Assays**—Two independent, well-established assays were used to measure KGDH activities. KGDH catalyzes the following reaction: α-ketoglutarate + NAD+ + CoA → succinyl CoA + CO2 + NADH. Both methods use the specific substrate α-ketoglutarate (α-KG) to distinguish NADH production from KGDH to that from other enzymes (i.e. substrate specificity). One method assays activity in cells whereas the other assesses the activity in slightly permeabilized cells in which the mitochondria are nearly intact (in situ assay).

**KGDH Activity Assays**—Two independent, well-established assays were used to measure KGDH activities. KGDH catalyzes the following reaction: α-ketoglutarate + NAD+ + CoA → succinyl CoA + CO2 + NADH. Both methods use the specific substrate α-ketoglutarate (α-KG) to distinguish NADH production from KGDH to that from other enzymes (i.e. substrate specificity). One method assays activity in cells whereas the other assesses the activity in slightly permeabilized cells in which the mitochondria are nearly intact (in situ assay).
3 mM Na-KG, 0.75 mM nitroblue tetrazolium (NBT), and 0.05 mM phenazine methosulfate) was transferred to cells. An assay mix without CoA and α-KG was used for the blank. After 30 min of incubation at 37 °C, the reaction was stopped by washing the cells twice with HBSS. The dark blue formazan was solubilized with 250 μl of 10% SDS in 0.1 N HCl overnight, and OD570 was read using a SPECTRA MAX 250 microplate reader (Molecular Devices). The OD570 was normalized with protein contents determined using a BCA™ protein assay kit (Pierce).

**Growth Rate—**Cells (3 x 10^4) were seeded into 96-well plates, and growth was monitored. Cells were fixed after 4 h, 3 and 5 days growth by adding 50 μl of 50% trichloroacetic acid and incubating at 4 °C for 1 h. Fixed cells were then rinsed twice with double-distilled water. Once the plate was air-dried, cells were stained with 100 μl of 0.4% sulforhodamine B solution (Sigma-Aldrich) for 30 min to measure the total protein. Then, the wells were rinsed twice with 1% acetic acid. After the plate was air-dried, 200 μl of 10 mM Tris was added to solubilize the incorporated dye, and OD570 was measured in a SPECTRA MAX 250 microplate reader (Molecular Devices). The background was measured in wells incubated with growth medium without cells.

**Live/Dead Viability Assay—**Cells were allowed to grow until they were nearly confluent. Cells were detached with cell dissociation solution (Sigma-Aldrich) and seeded into a 96-well plate at a density of 6 x 10^3 cells/100 μl per well. After incubation in a 37 °C, 5% CO2 incubator for 2 h, a total of live/dead viability working reagents containing 2 μM calcein AM and 4 μM ethidium homodimer-1 (EthD-1) (Molecular Probes Inc.) were added to the wells. After a further 30 min of incubation in the 37 °C, 5% CO2 incubator, calcein AM/EthD-1-stained cells were analyzed using a SPECTRA MAX GEMINI XDS fluorescence microplate reader in a well scan mode (fill pattern) with the following settings: Ex525/Em525, 590-nm cutoff for live cells; Ex485/Em525, 515-nm cutoff for dead cells. The relative percentage of dead cells was presented as relative fluorescence units (RFU) obtained from dead cells divided by RFU obtained from both live and dead cells.

The effects of H2O2 on cell viability were also determined. Cells were dissociated, seeded into two 96-well plates (6 x 10^4 cells/100 μl/well) as described above and allowed to incubate in a 37 °C, 5% CO2 incubator for 2 h. After a 0.5- or 23.5-h incubation with 100 μM H2O2, an addition of 1.1 mM H2O2 and 100 μl of live/dead viability working reagents were added to the wells. Cells were incubated for another half hour. Fluorescent signals were obtained, and the relative percentage of dead cells was calculated as described in the previous paragraph.

**DCF-detectable ROS in Response to External Oxidant—**Cells grown in 100-mm culture dishes (near confluent) were trypsinized and seeded into a 96-well plate (25 μg/ml)-coated 96-well plates at 3 x 10^4 cells/well. After 5 days in culture, cells were washed once with 100 μl of balanced salt solution (BSS) (140 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 5 mM glucose, 10 mM HEPES, and 2.5 mM CaCl2, pH 7.4), then loaded with 100 μl of 10 μM 6-carboxy-2,7'-dichlorodihydrofluorescein diacetate, diacetoxymethyl ester (6-C-H2DCF-AM) (Molecular Probes Inc.) in BSS buffer. After 1 h at 37 °C, cells were rinsed once with 100 μl of Ca-free BSS buffer and treated with 44 and 88 μM H2O2 in Ca-free BSS buffer, the plate was read at 1, 5, 10, 15, 30, and 60 min at an excitation wavelength of 485 nm and emission wavelength of 538 nm (cutoff 590 nm) using the SPECTRaman Gemini XDS fluorescence microplate reader. Basal DCF-detectable ROS was obtained with cells incubated with BSS buffer only.

**RESULTS**

**Generation and Characterization of the Stable Cell Lines—**E2k sense and antisense DNA in the expression vectors were confirmed by automatic DNA sequencing before the CsCl purification. The Flp-In-293 cell line was co-transfected with the expression vectors and pOG44 as described under "Experimental Procedures." To determine the role of an enzyme, particularly one that is part of an enzyme complex, a single clone or multiple clones with the same activities may give a misleading conclusion. Thus, at least 15 clones were screened for the effects of expression of E2k antisense RNA in mRNA and protein of E2k. Both mRNA and protein contents of E2k showed various levels of reduction in these clones. Cells with different levels of E2k protein are required to better understand the roles of E2k in the overall KGDHC activity and the responses to oxidative stress. We intentionally selected two clones to study in detail, one that had a reduction in E2k mRNA levels of about one-third and one with a reduction of about two-thirds. Different levels of reductions in mRNA are most likely because of the amount of antisense RNA expressed. However, there are still many other factors that may play important roles in determining the effectiveness of a particular antisense RNA, such as the half-life of the antisense RNA in vivo and the turnover time of the target mRNA.

The expression of an E2k antisense RNA in the chosen clones are shown in Fig. 1. The levels of E2k mRNA in these two clones were reduced to about 67% (E2k-mRNA-67) and 30% (E2k-mRNA-30) of the cells transfected with an E2k sense construct (E2k-mRNA-100) after normalization with β-actin (Fig. 2). Values in Fig. 2B represent mean relative densities from two independent experiments.

The protein levels of E1k, E2k, and E3 in E2k-mRNA-67 and E2k-mRNA-30 cells were examined by Western blotting with immunodetection. Proteins from cell lysates of E2k-mRNA-100, E2k-mRNA-67, and E2k-mRNA-30 were separated by SDS-PAGE and transferred to nitrocellulose membranes and probed with antibodies to the three subunits and β-actin (as internal control) as described under "Experimental Procedures." E2k antisense RNA expression reduced E2k protein in E2k-mRNA-67 and E2k-mRNA-30 cells to about 46 and 23% of the sense control (E2k-mRNA-100) after normalization with β-actin (Fig. 3). The protein levels of the three subunits in these two cell clones are summarized in Table I.

**KGDH Activity—**Consequences of varied E2k proteins on overall KGDHC activities were examined by two methods. KG-DHC activities of E2k-mRNA-67 and E2k-mRNA-30 in the cell lysates as measured by α-KG-dependent NADH generation assay were reduced by ~48% (E2k-mRNA-67 and 10% (E2k-mRNA-30) compared with E2k-mRNA-100 (Fig. 4A). Similar changes were observed when KG-DHC was measured with an in situ histochemistry assay. This assay assesses KG-DHC activities within mitochondria of cells that have been permeabilized enough to allow substrate and cofactor penetration. KG-DHC activities of E2k-mRNA-67 and E2k-mRNA-30 diminished by about 45 and 10% compared with E2k-mRNA-100 (Fig. 4B).

**Growth Rate—**The effect of different levels of diminished E2k on cell growth was tested. Growth rates of E2k-mRNA-67 and E2k-mRNA-30 cells were significantly reduced about 50%
Interestingly, when cells were incubated with 100 dead cells in E2k-mRNA-30 increased 68% (Fig. 6). E2k-mRNA-100 cells increased about 30%, while the percent of cell death in all three lines. Cell death in E2k-mRNA-67 and DNA ladder. A PCR. RNA-expressing cells as evaluated by semiquantitative RT-

A difference in viability (Fig. 6). tested. Under normal conditions, cells showed no significant difference in viability (Fig. 6). Effects of Expression of E2k Antisense RNA on Cell Viability under Normal Conditions and Following Oxidative Stress—Whether the partial reduction of E2k subunit altered cell viability or made the cells more sensitive to oxidative stress was tested. Under normal conditions, cells showed no significant difference in viability (Fig. 6A).

Viabilities in response to oxidative stress induced by H2O2 were also examined. Cells were incubated with 100 μM H2O2 in either BSS with just glucose as substrate or D-PBS buffer with glucose and pyruvate as substrates for either 1 or 24 h, and cell death was assessed. Diminished E2k did not alter the response to H2O2 at 1 h in either buffer (Fig. 6B). However, the treatment of cells with 100 μM H2O2 in BSS buffer for 24 h increased cell death in all three lines. Cell death in E2k-mRNA-67 and E2k-mRNA-100 cells increased about 30%, while the percent of dead cells in E2k-mRNA-30 increased 68% (Fig. 6B, left panel). Interestingly, when cells were incubated with 100 μM H2O2 in D-PBS buffer with glucose and pyruvate as substrates for 24 h, the percent increase of dead cells was only 27% for E2k-mRNA-30, 16, and 18% for E2k-mRNA-100 and E2k-mRNA-67, respectively (Fig. 6B, right panel).

DCF-detectable ROS in Response to External Oxidant—The ability of cells with different levels of E2k to handle oxidative stress induced by H2O2 was demonstrated by monitoring the oxidation of c-H2DCF as described under “Experimental Procedures.” Addition of H2O2 increased DCF-detectable ROS significantly in all cell lines. The H2O2-induced increase was significantly greater in the E2k-mRNA-30 line with the least E2k content (Fig. 7). H2O2 (44 μM) increased DCF signal significantly more in E2k-mRNA-30 than in E2k-mRNA-100, whereas no difference was observed between the E2k-mRNA-67 and E2k-mRNA-100 cells (Fig. 7A). Higher H2O2 (88 μM) concentration produced a similar pattern. After the cells were incubated with 88 μM of H2O2 for 60 min, the DCF signal was 10.0 ± 0.53 (E2k-mRNA-30) and 6.2 ± 0.25 (E2k-mRNA-100), respectively (Fig. 7B). On the other hand, no significant increase in DCF signal was observed at 60 min in E2k-mRNA-67 cells (7.1 ± 0.35) compared with E2k-mRNA-100 cells (Fig. 7B). The results suggested a significant reduction in diminishing of H2O2-induced DCF-detectable ROS in E2k-mRNA-30 cells.

**DISCUSSION**

A partial reduction in KGDHC activity occurs in brains of individuals with AD (1–4) and a number of other neurodegenerative disorders (5–11). The subunits may be selectively altered. For example, brains from AD patients bearing the APP670/671 mutation have reductions in overall KGDHC ac-
activities (−55% to −57%) and in the immunoreactivities of E1k (−51%) and E2k (−76%) but not E3 (15). Oxidative stress also selectively alters the immunoreactivity of the subunits. Peroxynitrite (ONOO⁻) diminishes activity and reduces immunoreactivity of E1k and E2k, while nitric oxide (NO⁻) reduces KGDHC activity without changing the immunoreactivity of any of the three subunits (32). Thus, further studies on the consequences of partially diminished E1k or E2k in mammalian cells will help us to better understand the role of KGDHC in cell function and neuronal death. The studies in this manuscript focused on the role of E2k.

Genetic manipulation of E2k has not been reported previously in mammalian cells. The only reports in mammalian systems for genetic manipulation of KGDHC are studies on E3.
which is common to all α-ketoacid dehydrogenase complexes (37). The null mutation in the murine E3 gene causes perigasttrulation lethality, but the heterozygotes survive (37). Heterozygotes of E3 reduce KGDHC by about one-half in brains and livers (38). The present results shows that a reduction of E2k mRNA in HEK293 cells using an antisense RNA strategy causes a proportional decline in E2k protein. The protein levels of E1k and E3 were not diminished. Indeed, in the clone with a 77% decrease in E2k protein, E1k protein increased by 40% (see Table I). More data will be required to reach conclusions about regulation or compensation. The mechanism for the increase in E1k when E2k was diminished below a critical limit is unknown. In yeast, the complete deletion of E2k causes no change in mRNA for E1k or E3 but neither protein levels nor KGDHC activities were determined (39), and this has not been studied in mammalian cells. Thus, the HEK293 cell lines allowed us to test the role of E2k in the function of KGDHC and cell function.

One of the most surprising results was that the reductions in the level of E2k protein did not produce a proportional decline in the activity of the KGDHC complex. Although a 54% reduction in E2k protein was associated with a 48% reduction in KGDHC activity, a 77% reduction in E2k protein was associated with only a 10% reduction in KGDHC activity. As described in the introduction, KGDHC is composed of multiple copies of each subunit. The specific activities of the E1k and E2k subunits have not been determined in cell lysates. An in vitro study of the isolated components from KGDHC from pig heart shows that the specific activity of E1k and E2k are 144 and 76 μmol/h/mg protein, respectively (40). However, KGDHC is a multiple enzyme complex that consists of multiple copies of the three subunits that catalyze sequential reactions. Thus, the above values for isolated components may not reflect the activities of the subunit within the complex. In fact, E2k is suggested to be more active than E1k (41–43). For example, an early study on kinetics of succinylation (E1k) and desuccinylation (E2k) of KGDHC isolated from E. coli suggests that desuccinylation of the complex by E2k is more rapid than E1k catalyzed succinylation as indicated by the greater rate constant of 200 s⁻¹ compared with 49 and 89 s⁻¹ for succinylation (42). Taken into account of E2k is more active than E1k, the most reasonable speculation about the data in the current study is that the decline in KGDHC activity stimulated production of more E1k (41–43). For example, an early study on kinetics of succinylation (E1k) and desuccinylation (E2k) of KGDHC isolated from E. coli suggests that desuccinylation of the complex by E2k is more rapid than E1k catalyzed succinylation as indicated by the greater rate constant of 200 s⁻¹ compared with 49 and 89 s⁻¹ for succinylation (42). Taken into account of E2k is more active than E1k, the most reasonable speculation about the data in the current study is that the decline in KGDHC activity stimulated production of more E1k (41–43).
they interact in vivo to form the functional complexes under various situations. Some relevant studies in yeast have tested the responses of the other two subunits and assembly in the absence or in the presence of excess of E2k (39, 45–46). However, these studies were only with a total deletion of E2k or overexpression. The overall KGDHC activity is reduced when KGDHC is assembled in the presence of excess of E2k (46). Whereas in the absence of E2k, separated dimeric E1k and E3 are detected, activity was not measured (45). The possible existence of heterogeneous quaternary structures of /H9251-ketoacid dehydrogenase complexes (47–50) implies the potential flexibility in subunits composition of KGDHC and its response to challenges. Unlike a simple group of oligomeric enzymes, the KGDHC is very dynamic, and that may underlie the lack of parallelism between E2k content and the overall KGDHC activity. One of the interesting observations from the same study mentioned in the previous paragraph (42) shows that when the E1k component is partially inhibited by maleimide, the remaining active E1k succinylates more lipid acids than the unmodified enzyme complex. This again suggests that the enzyme complex is very dynamic in its response to challenges. However, no other detailed kinetics studies are available on how the complex responds to the inhibition of E2k component. Furthermore, the responses of the complex to different challenges within cells would be more complicated. In vitro reconstitution of KGDHC with varied components of the three subunits would provide evidence to further understand how the functional complexes are assembled and how that would result in relevant changes in the complex activities, particularly in the case of diminished E2k. However, this would not necessarily mimic the results within the mitochondria in cells. Regardless of the source of the paradox between KGDHC activity and E2k contents, the divergence provides an excellent model to test whether E2k protein or KGDHC activities are more highly correlated to physiological responses.

The unexpected return to near normal KGDHC activity in E2k-mRNA-30 provided the possibility of testing whether physiological changes were related to KGDHC activities or E2k protein levels. Changes in three physiological responses caused by a reduction in E2k mRNA were more highly correlated to a loss E2k protein than to KGDHC activity. A reduction in growth rate was associated with a decline in E2k protein and not with complex activity. Both clones with reduced E2k protein grew about 50% slower than the control even though one of them had nearly normal KGDHC activity. One possibility is that the cells were growing in complete media with high glucose so that the energy was supplied by glycolysis. Thus, KGDHC was not likely to be required for metabolic purposes, but was required for its other functions (e.g. transferring reducing equivalents to the right proteins such as thioredoxin). A reduction in E2k protein altered the ability of the cells to handle external oxidants. To test whether E2k plays an important role in reaction to oxidative stress, the response to H2O2 was determined in the two clones with varying levels of E2k. H2O2 produced more cell death in E2k-mRNA-30 cells, with a 77% reduction in E2k protein, than in the E2k-mRNA-67 or E2k mRNA-100 cells (Fig. 6). A significantly greater H2O2-induced DCF-detectable ROS was also observed in E2k-mRNA-30 cells (Fig. 7). An analogous result occurs in vivo in E3+/− heterozygotes. The brains of these mice have increased concentrations of malondialdehyde (a marker of oxidative stress). Furthermore, both the cell death and markers of oxidative stress in response to neurotoxins are exaggerated (38). However, E3 is common to all α-keto acid dehydrogenases. Thus, the activities...
of KGDC and pyruvate dehydrogenase are both diminished. Furthermore, the protein levels of E1k and E2k have not been determined in these mice. Thus, the present study suggested that growth rate, and the oxidant-induced change in ROS and cell death are more highly correlated to E2k protein level than to KGDC activities.

Expanding evidence suggests that the enzymes of the tricarboxylic acid cycle may have roles beyond those of just generating NADH for the electron transport chain. Enzymes such as aconitase, isocitrate dehydrogenase and Kgd2p (a subunit of KGDC in yeast equivalent to E2k in mammal), are found to have two or more different functions (51–56). For example, Kgd2p in yeast participates in both the tricarboxylic acid cycle and ρ+ mtDNA maintenance (56). Thus, E2k might play an additional role in handling cellular oxidative stress beyond its catalytic function in the KGDC complex. KGDC has a supporting role in oxidative defenses. For example, in mycobacteria (Mtbb) (35), the E3 and E2 components of α-ketocarboxylase dehydrogenase complexes mediate electron flow from NADH to peroxiredoxin alkyl hydroperoxide reductase (AhpC) through a thioredoxin-like protein (AhpD). A possible indirect functional association of SP-22, a member of the peroxiredoxin (Prx) family, to E3 via thioredoxin (Trx) system and E2k also occurs (57). As an important component of one of the endogenous antioxidant systems (58–59), Trx undergoes NADPH-dependent reduction by Trx reductase and has also been shown to directly participate in the regulation of KGDC (60–62). Taken together, we speculate that E2k can participate in cellular antioxidant defense systems through Trx. Therefore, cells with E2k content below certain limits could not handle oxidative stress as well as greater accumulation of ROS in E2k-mRNA-30 cells. This occurred even though overall activity of the complex was near normal.

In response to limited NADPH under oxidative stress (63–64), the E2k dihydrolipoamide intermediate is likely to contribute partially in recycling oxidized Trx, in addition to its reoxidation by E3 for the NADH production. In this case, E2k would act as a Trx reductase-like protein. In addition to its role in antioxidant defense system, the reduced form of Trx acts as a growth factor, and inhibition of Trx reductase is associated with diminished cell growth (65–70). Interestingly, both E2k-mRNA-67 and E2k-mRNA-30 cells showed diminished growth (Fig. 5). The oxidative stress could push a portion of E2k to the Trx-dependent antioxidant defense pathway and limit production of NADH, which is associated with declined overall KGDC activity. Involvement of glutaredoxin system in KGDC inactivation through glutathionylation of KGDC provides another mechanism for KGDC to interact with cellular thiol redox state (71).

In summary, we successfully generated HEK293 cell clones with different levels of deficiency in the E2k subunit of KGDC. Reductions in E2k protein did not always predict effects on activities of the whole complex. Physiological measurements including the response to oxidative stress more closely paralleled changes in E2k protein than the activity of the complex. These findings suggest multiple roles of E2k under oxidative stress, and raise the possibility of the involvement of the E2k subunit of KGDC in cellular antioxidant defense systems, in addition to its normal function of generating NADH. Thus, in neurodegenerative diseases, a reduction in E2k may be critical for more reasons than just as a loss of activity of the KGDC complex.

Acknowledgment—We thank Dr. Soo-Youl Kim for help with the experimental approach.
W. H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 574–578
54. Jeffery, C. J. (1999) Trends Biochem. Sci. 24, 8–11
55. de Jong, L., Elzinga, S. D., McCammon, M. T., Grivell, L. A., and van der Spek, H. (2000) FEBS Lett. 483, 62–66
56. Kaufman, B. A., Newman, S. M., Hallberg, R. L., Slaughter, C. A., Perlman, P. S., and Buitow, R. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7772–7777
57. Gourlay, L. J., Bhella, D., Kelly, S. M., Price, N. C., and Lindsay, J. G. (2003) J. Biol. Chem. 278, 32631–32637
58. Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994) J. Biol. Chem. 269, 27670–27678
59. Chae, H. Z., Robison, K., Poole, L. B., Church, G., Storz, G., and Rhee, S. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7017–7021
60. Bunik, V., Vollmann, H., and Bisswanger, H. (1997) Biol. Chem. 378, 1125–1130
61. Bunik V. (2000) J. Mol. Catalysis B: Enzymatic 8, 165–174
62. Bunik, V. I. (2000) Eur J. Biochem. 270, 1036–1042
63. Asahina, T., Kashiwagi, A., Nishio, Y., Ikebuchi, M., Harada, N., Tanaka, Y., Takagi, Y., Saeki, Y., Kikkawa, R., and Shigeta, Y. (1995) Diabetes 44, 520–526
64. Chinopoulos, C., Trettter, L., and Adam-Vizi, V. (2000) Neurochem. Int. 36, 483–488
65. Nakasugi, N., Tagaya, Y., Nakasugi, H., Mitsu, A., Maeda, M., Yodsi, J., and Tursz, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 97, 8282–8286
66. Oblong, J. E., Berggren, M., Gasdaska, P. Y., and Powis, G. (1994) J. Biol. Chem. 269, 11714–11720
67. Gasdaska, J. R., Berggren, M., and Powis, G. (1995) Cell Growth Differ. 6, 1643–1650
68. Mustacich, D., and Powis, G. (2000) Biochem. J. 346, 1–8
69. Khayat, M., Stuge, T. B., Wilson, M., Bengten, E., Miller, N. W., and Clem, L. W. (2001) J. Immunol. 166, 2937–2943
70. Kim, M. R., Chang, H. S., Kim, B. H., Kim, S., Baek, S. H., Kim, J. H., Lee, S. R., and Kim, J. R. (2003) Biochem. Biophys. Res. Commun. 304, 119–124
71. Oulton-Persson, A. C., Starke, D. W., Mieyal, J. J., and Szweda, L. I. (2003) Biochemistry 42, 4235–4242
Reduction in the E2k Subunit of the α-Ketoglutarate Dehydrogenase Complex Has Effects Independent of Complex Activity
Qingli Shi, Huan-Lian Chen, Hui Xu and Gary E. Gibson

J. Biol. Chem. 2005, 280:10888-10896.
doi: 10.1074/jbc.M409064200 originally published online January 12, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M409064200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 68 references, 23 of which can be accessed free at
http://www.jbc.org/content/280/12/10888.full.html#ref-list-1