

Selenium deficiency increases susceptibility to glutamate-induced excitotoxicity¹

NICOLAI E. SAVASKAN,^{*,§,2,3} ANJA U. BRÄUER,^{*,3} MARKUS KÜHBACHER,[†]
ILKER Y. EYÜPOGLU,^{*,‡} ANTONIOS KYRIAKOPOULOS,[†] OLAF NINNEMANN,^{*}
DIETRICH BEHNE,[†] AND ROBERT NITSCH^{*}

^{*}Institute of Anatomy, Department of Cell and Neurobiology, [§]Department of Neurology, Humboldt University Medical School Charité, D-10115 Berlin, Germany; [†]Hahn-Meitner-Institute, Department Molecular Trace Element Research in the Life Sciences, D-14109 Berlin, Germany; and [‡]Department of Neurosurgery, University of Erlangen-Nürnberg, D-91054 Erlangen, Germany

SPECIFIC AIMS

Excitotoxic brain lesions, such as stroke and epilepsy, lead to increasing destruction of neurons hours after the insult. The deadly cascade of events involves detrimental actions by free radicals and the activation of proapoptotic transcription factors, which finally results in neuronal destruction. We provide direct evidence that the essential trace element selenium has a pivotal role in neuronal susceptibility to excitotoxic lesions and seizure activity.

PRINCIPAL FINDINGS

1. Selenium prevents glutamate-induced cell death

We first treated the hippocampal neuronal cell line HT22 with selenium in form of selenite (Na_2SeO_3). Selenite concentrations of up to the micromolar range were tolerated by neurons without any influence on cell viability, whereas higher amounts of selenium led to cell death (Fig. 1a). Excitotoxic conditions were induced in neurons by treatment with an excess of glutamate, the most abundant excitatory neurotransmitter in the brain. Glutamate treatment reduced cell survival by > 80%. This glutamate-induced cell death could be prevented by simultaneous application of selenite in a concentration-dependent manner (Fig. 1b–d). The calculated median effective concentration (EC_{50}) of selenite was 37 nM with a maximal effect (98% protection) at 100 nM, which is within the physiological range as shown in human studies in vivo. Glutamate-induced cell death could be prevented in rescue experiments where selenite was added hours after glutamate damage (Fig. 1e).

2. Selenium inhibits NF- κ B and AP-1 activation and requires protein synthesis

To further elucidate the underlying mechanisms of selenium-mediated neuroprotection, we performed measurements of peroxide and glutathione levels. We found that an excess of glutamate in neurons induced high levels of peroxides, which was prevented by selenite treatment, whereas there was no effect by selenium on the altered glutathione levels. Next, we used specific blockers of the

cystine/glutamate antiporter (10 mM homocysteate) and glutathione synthetase (200 μM buthionine sulfoximine), both of which induced glutathione depletion, subsequently resulting in cell death. These findings indicate that the mode of action of selenium is independent of lowered glutathione levels. Protective effects were achieved when selenite was washed out 2 h after glutamate treatment (70% protection) and by treatment with selenate (Na_2SeO_4), which in contrast to selenite lacks direct antioxidative properties but is like selenite incorporated in selenoproteins. The assumption that selenite neuroprotection requires protein synthesis was confirmed by the finding that inhibition of protein synthesis by cycloheximide abolished the protective selenium effects. Glutamate treatment led to increased intranuclear NF- κ B levels, which could be inhibited by increasing selenite concentrations. Using gel shift assays, we showed that the glutamate-induced activation and binding of NF- κ B and AP-1 to their nuclear response elements in hippocampal HT22 cells was decreased by selenium. Since we did not detect any direct effect of selenium on NF- κ B binding activity, it appears that selenium in the form of selenium-containing proteins is involved in antiapoptotic mechanisms. HT22 cells do not express functional glutamate receptors, and glutamate toxicity is therefore mediated by inhibiting the cystine uptake. To test the role of selenium on glutamate receptor-mediated excitotoxicity, we applied selenite onto a glutamate receptor bearing primary hippocampal neurons. Selenite was still effective in reducing neuronal cell death by ~60%. To assess this protective potential of selenium in neurons maintained in their organotypic environment, we applied glutamate to hippocampal slices. Cell death was substantially decreased by selenite treatment under these in vitro conditions. These findings indicate that receptor-independent oxidative glutamate toxicity is substantially attenuated by selenium not

¹ To read the full text of this article, go to <http://www.fasebj.org/cgi/doi/10.1096/fj.02-0067fje>; to cite this article, use *FASEB J.* (November 1, 2002) 10.1096/fj.02-0067fje

² Correspondence: Institute of Anatomy, Department of Cell Biology and Neurobiology, (contract SPP322-1087) Humboldt University Medical School Charité, 10098 Berlin, Germany. E-mail: nicolai.savaskan@charite.de

³ Both authors contributed equally to this work.

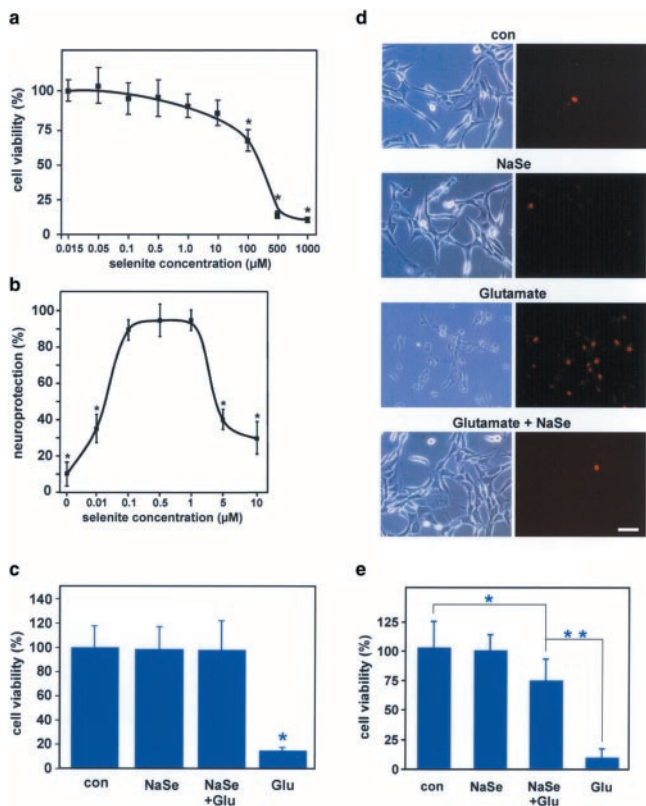


Figure 1. Neuroprotection and oxidative stress is attenuated by selenium. *a*) Concentration-dependent effect of selenium added in the form of sodium selenite (Na_2SeO_3 , abbreviated as Se) on HT22 cell viability. Neurons were treated with Se for 24 h and cell viability was assayed with MTT. The basal selenium levels in control conditions were 15 nM. Neuronal cell survival was expressed as % of neuroprotection to controls set at 100%. Measure points represent mean \pm SD of 5 experiments run in triplicate. *b*) Concentration-dependent neuroprotection of selenium in glutamate treatment. 10 mM glutamate was applied to the cells with subsequent addition of selenite (Se) at different concentrations. *c*) Data representing HT22 cells treated with 10 mM glutamate and 100 nM selenite for 24 h. *d*) Bright-field (left) and rhodamine fluorescence (right) micrographs of the experiment illustrated in panel *c*. PI-positive (red) cells indicate cell death. Scale bar, 8 μm . *e*) Rescue experiments in which cells were treated for 2 h with glutamate before selenite was added. Asterisks denote significant differences from untreated controls (mean \pm SD, * $P < 0.01$; Mann-Whitney *U* test).

only in neuronal cell lines, but also achieves significant neuroprotection in primary hippocampal neurons and hippocampal brain slices.

3. Selenium deficiency results in massive increase in susceptibility and cell damage

To finally prove that these neuroprotective effects of selenium observed in vitro are relevant in an in vivo model of excitotoxicity, we performed studies of rats provided with either a selenium-adequate or -deficient diet. Because of the hierarchy in the selenium distribution in selenium deficiency, selenium concentrations in the liver were dramatically reduced in animals on the selenium-deficient diet

whereas selenium content in the brain remained high, though nevertheless significantly reduced by $\sim 10\%$ (Fig. 2a). The brain in selenium-deficient rats had no obvious

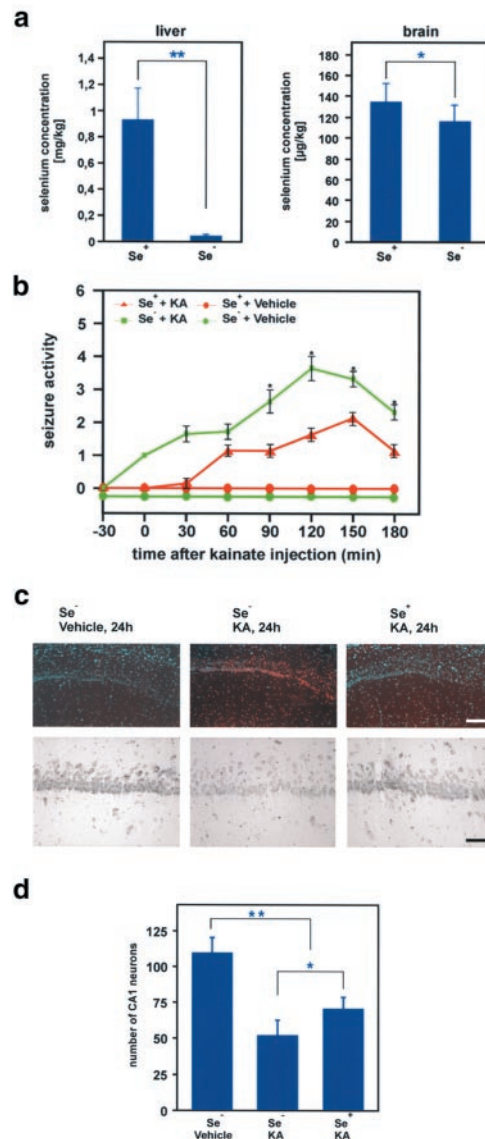


Figure 2. Selenium deficiency increases seizure activity and cell damage. *a*) Selenium concentration of selenium-deficient (Se^-) and selenium-adequate (Se^+) rats in the liver and brain. *b*) Time course of seizure activity after i.p. applied kainate (KA) or saline (Vehicle). Data denotes seizure scores (staring, freezing, and mouth/facial twitchings were scored as 1; rolling and wild running as 6). Scores at various times after kainate injections were analyzed by two independent observers and averaged and compared by two-tailed *t* tests (means \pm SD). *c*) Enhanced seizure-induced caspase-3 staining (upper panel; red, caspase-3 staining; blue, nuclear staining) and cell death in hippocampus of selenium-deficient rats (Se^-). Photomicrographs of caspase-3-stained (upper panel) and Nissl-stained brain sections (lower panel) from animals with i.p. applied kainate (KA) or saline (Vehicle) taken 24 h after injection. Note the marked staining of CA1 pyramidal neurons for caspase-3 and loss of Nissl-positive neurons in selenium-deficient rats. *d*) Bars denote numbers of Nissl-positive neurons. * $P < 0.01$; ** $P < 0.001$ (mean \pm SD, two-tailed *t* test with Bonferroni correction for multiple comparisons). Scale bar: *c*) 80 μm (upper panel), 25 μm (lower panel).

macroscopical and histological phenotypic differences from that of the rats on a selenium-adequate diet. In the kainate model of excitotoxicity, however, the reduction in brain selenium levels was sufficient to result in a remarkably higher seizure rate compared with animals on a selenium-adequate diet (Fig. 2*b*). Under these excitatory conditions, we found an increased number of caspase-3-positive cells in selenium-deficient rats, and neuronal cell loss in the hippocampus was significantly higher than rats on a selenium-adequate diet (Fig. 2*c, d*). Taken together, we show here that selenium deficiency increases susceptibility to kainate-induced excitotoxicity resulting in seizure activity and cell damage *in vivo*.

CONCLUSIONS

This study was designed to determine the potential capacity of selenium in the protection of neurons under circumstances of excitotoxic insults. Selenium intake levels are considered inadequate in many regions throughout Europe and North America. The fact that the brain retains selenium even after long-term selenium deprivation indicates the importance of this trace element to normal brain function. Intractable epileptic seizures in children with low blood selenium concentrations showed a substantial improvement in clinical state and EEG's with oral selenium substitution. Therefore, we tested the effect of selenium deficiency using an *in vivo* model of kainate-induced excitotoxicity. We could show that selenium-deficient rats were more susceptible to kainate-induced excitotoxicity which resulted in a higher seizure rate than with selenium-adequate fed controls. In addition, we detected a significantly higher loss of hippocampal neurons in the selenium-deficient cohort, indicating severe structural damage under these conditions.

Selenium deficiency might not only affect neurons but also glial cells in the brain. One major player appears to be the microglial cell population, which is known to increase primary neuronal damage dramatically during the first days after insult (Fig. 3). Selenium also has anti-inflammatory properties involving the cyclo-oxygenase and lipoxygenase pathways affecting cytokine and chemokine expression. Our present findings reveal that protection against the primary damage crucially depends on selenium levels in the brain (Fig. 3). In fact, our data suggest that the survival-promoting selenium actions are achieved in the early phase of the excitotoxic event directly after insult. We could demonstrate that selenium protects neurons in a wide physiological range before becoming toxic. This protective effect is not mediated directly by antioxidative effects of selenite as is the case for the organic selenium compound ebselen. Therefore, *de novo* protein synthesis is necessary for selenium-mediated neuroprotection. It has been shown that with insufficient selenium intake there is a hierarchy in the incorporation of selenium into the different selenoproteins. In all tissues (including the brain), cellular glutathione peroxidase is last in this hierarchy. Thus, the decrease in concentration of

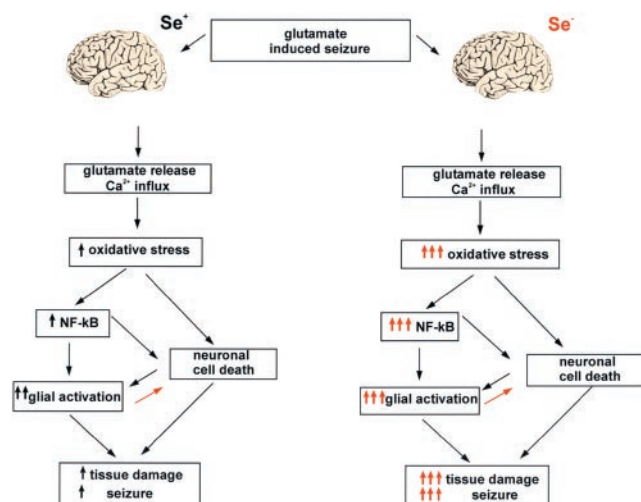


Figure 3. Schematic summary and hypothesis of the effects of selenium deficiency on the central nervous system. The situation under adequate selenium supply (left side) and selenium-deficient (right side) is shown. Selenium-deficiency leads to increased oxidative stress with subsequent NF- κ B and AP-1 activation and neuronal cell death. Since we focused on the primary neuronal cell damage under excitotoxic conditions, the red arrow indicates the link between glial activation and neuronal cell death. Whether selenium acts on glial cells as well is so far unknown. Future studies will extend this study to secondary cell damage triggered by activated microglial and astroglial cells.

this glutathione-dependent enzyme is more pronounced than that of the other selenoproteins, indicating a role in the susceptibility to neuronal damage under selenium-deficient conditions. One could suppose that under physiological selenite treatment, expression of a hitherto unknown selenoprotein is induced. It has been shown in differentiating oligodendroglial cells that selenium induces the expression of non-selenoproteins such as MBP, MAG, and PLP. These findings indicate that selenium in the form of selenite does not act as another free radical scavenger (as known for α -tocopherol or ebselen). Inhibition of the nuclear translocation of NF- κ B and AP-1 binding by selenium indicates the involvement of selenium in protein-dependent anti-apoptotic mechanisms. Preventive strategies against excitotoxic brain damage should therefore not rely solely on general substitution of well-known antioxidative agents, but must specifically consider selenium levels as an independent factor. This is of clinical relevance since a significant cell death rate in seizure, ischemia, and CNS trauma is independent of glutamate receptors. Taken together, our data demonstrate that selenium levels within the physiological range attenuate excitatory amino acid damage. Therefore, adequate selenium supplementation is an important strategy to prevent the detrimental effects of excitotoxicity and can even be effective early after the initial insult. These results render selenium a powerful preventive and therapeutic substance to excitotoxic brain damage distinct from known antioxidative substances.

[E]