

# Endothelial Effects of 3-Hydroxyglutaric Acid: Implications for Glutaric Aciduria Type I

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**ABSTRACT:** Infants with glutaric aciduria type 1 (GA1) are subject to intracranial vascular dysfunction. Here, we demonstrate that the disease-specific metabolite 3-hydroxyglutaric acid (3-OH-GA) inhibits basal and vascular endothelial growth factor (VEGF)-induced endothelial cell migration. 3-OH-GA affects the morphology of VEGF-induced endothelial tubes *in vitro* because of partial disintegration of endothelial cells. These effects correlate with VE-cadherin loss. Remarkably, 3-OH-GA treatment of human dermal microvascular endothelial cells leads to disruption of actin cytoskeleton. Local application of 3-OH-GA alone or in combination with VEGF in chick chorioallantoic membrane induces abnormal vascular dilatation and hemorrhage *in vivo*. The study demonstrates that 3-OH-GA reduces endothelial chemotaxis and disturbs structural vascular integrity *in vitro* and *in vivo*. These data may provide insight in the mechanisms of 3-OH-GA-induced vasculopathic processes and suggest N-methyl-D-aspartate receptor-dependent and -independent pathways in the pathogenesis of GA1. (*Pediatr Res* 59: 196–202, 2006)

GA1 is a neurometabolic disorder caused by deficiency of the mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) and affects the catabolism of L-lysine, hydroxy-lysine, and L-tryptophan, leading to accumulation of GA, 3-OH-GA, and glutaconic acids in body fluids of affected patients. Distinct clinical manifestations typically occur in the first 18 mo of life, when a nonspecific infectious illness or routine vaccination precipitates acute stroke-like striatal degeneration resulting in dystonic-dyskinetic movement disorder (1,2). A great variability in clinical presentations has been observed without clear genotype-phenotype correlation (3,4).

The pathomechanisms in GA1 leading to neuronal death are still not understood and are presumably multifactorial. Due to structural relation of 3-OH-GA with glutamate, it has been proposed that 3-OH-GA may act *via* interaction with NMDA receptors, resulting in excitotoxic neuronal damage (5–7).

Up to 30% of GA1 patients develop subdural hematomas that generally are thought to be consequence of ruptures of stretched veins due to frontotemporal hypoplasia (5,8).

Whereas no morphologic changes in vasculature of brain autopsy material of six North American aboriginals with GA1 aged 8 mo to 40 y have been observed (9), MRI examinations of GA1 patients show chronic extravasations from transarachnoid vessels independent of an encephalopathic crisis, as well as abnormally extended intrastriatal vessels with perivascular hyperintensities observed during an encephalopathic crisis, imposing as local vasogenic rather than cytotoxic edema (K. Strauss, personal communication). To investigate mechanisms influencing endothelial function, the effects of 3-OH-GA were studied on HDMEC *in vitro* and on chick CAM *in vivo*.

## METHODS

**Materials.** VEGF<sup>165</sup> was purchased from R & D Systems (Minneapolis, MN). The following antibodies were used: monoclonal anti-vimentin (DAKO, Copenhagen, Denmark), polyclonal anti-VE-cadherin, anti-occludin, anti-VEGF, anti-VEGFR-1 and -2 (SantaCruz Biotechnology, Santa Cruz, CA); secondary biotin (immunohistochemistry) and horseradish peroxidase (HRP) (Western blotting)-conjugated antibodies (Jackson ImmunoResearch, West Grove, PA), TRITC-conjugated phalloidin (Dianova, Hamburg, Germany). GA,  $\alpha$ -ketoglutaric, and D2-hydroxyglutaric acids were purchased from Sigma Chemical Co. (St. Louis, MO). 3-OH-GA was synthesized from dimethyl- $\beta$ -ketoglutarate (Sigma Chemical Co.) by NaBH<sub>4</sub> reduction followed by ester-cleavage by KOH.

**Endothelial cell culture and treatment.** HDMEC (PromoCell, Heidelberg, Germany) were cultured as described previously (10). At confluency, cells were either incubated in presence or absence of effectors for 24 h at 37°C.

**Endothelial cell migration assay.** Endothelial cell migration assay was carried out as described previously (10).

**Endothelial tube formation assay.** Endothelial tube formation assay was performed as described previously (10,11).

**SDS-PAGE and Western blots** Extracted proteins (25  $\mu$ g) were separated by SDS-PAGE, blotted and incubated using anti-VE-cadherin antibody (final dilution: 1:100) and HRP-conjugated anti-rabbit IgG (1:10,000), or anti-vimentin antibody (final dilution: 1:400) and visualized by ECL (Pierce Biotechnology, Inc., Rockford, IL).

**Immunohistochemistry.** Paraffin sections from collagen gels of tube formation assays were used for immunohistochemistry as described previously (10,12). Immunostaining was performed using anti-VEGF (1:400), -VEGFR-1 (1:200), -VEGFR-2 (1:200), -occludin (1:80), and -VE-cadherin (1:50) antibodies, biotin-conjugated secondary antibodies, ABC-complex (DAKO), and the Ni-enhanced glucose oxidase detection system (13). As

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**Abbreviations:**  $\alpha$ -KGA,  $\alpha$ -ketoglutaric acid; CAM, chick chorioallantoic membrane; D2-OH-GA, D-2-hydroxyglutaric acid; GA, glutaric acid; GA1, glutaric aciduria type 1, *McKusick 231670*; HDMEC, human dermal microvascular endothelial cells; NMDA, N-methyl-D-aspartate; 3-OH-GA, 3-hydroxyglutaric acid; TRITC, tetramethylrhodamine isothiocyanate; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

controls, primary and/or secondary antibodies were replaced by buffer, or sections were incubated with normal rabbit serum (Sigma Chemical Co.) instead of primary antibody.

**Fluorescence staining of actin cytoskeleton.** HDMEC were cultivated in eight-well chamber slides until subconfluency and incubated with 3-OH-GA at indicated concentrations for 24 h. After washing, cells were fixed for 20 min with 4% paraformaldehyde, washed again, and incubated for 60 min with TRITC-conjugated phalloidin (1:100). Subsequently, cells were washed twice and processed for digital fluorescence microscopy (Zeiss, Jena, Germany).

**Chick CAM assay.** CAM assay was carried out as described previously (10,14).

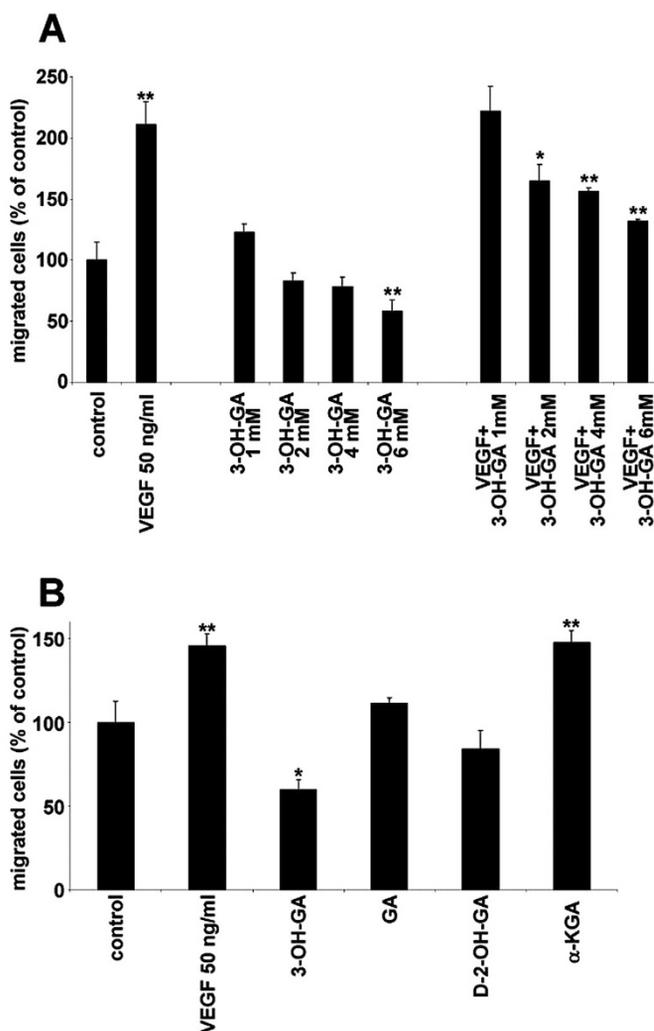
**RT-PCR analysis.** Five hundred nanograms total RNA, prepared from HDMEC and tissue samples as described previously (15), were used as template for RT-PCR analyses, using GeneAmp RNA PCR kit (PerkinElmer Life Science, Boston, MA) according to the manufacturer's instructions.

**Data analysis.** Data were analyzed using one-way ANOVA followed by Scheffé test. Significance was accepted at  $p < 0.05$ . SPSS 12.0 software (SPSS Inc., Chicago, IL) was used for calculations.

## RESULTS

**3-OH-GA inhibits endothelial migration.** To examine direct effects of 3-OH-GA on endothelial cells, migration of HDMEC was studied using a modified Boyden chamber (Fig. 1A). Treatment of cells with 2, 4, and 6 mM 3-OH-GA decreased the number of migrated HDMEC to 83%, 78%, and 58% of controls, respectively. VEGF (50 ng/mL), used as positive control for chemotactic migration, increased the number of migrating HDMEC about 2-fold. Simultaneous incubation of HDMEC with VEGF and 2, 4, and 6 mM 3-OH-GA led to a concentration-dependent significant reduction in the number of migrated cells to 78%, 74%, and 63% of controls, respectively. To determine the specificity of 3-OH-GA, migration assays were performed in the presence of 6 mM GA, D2-OH-GA, and  $\alpha$ -KGA (Fig. 1B). Only 3-OH-GA leads to a significant reduction of the number of migrated cells, whereas GA and D2-OH-GA did not impair the cell migration rate. Of note, in the presence of  $\alpha$ -KGA the number of migrated cells was significantly increased.

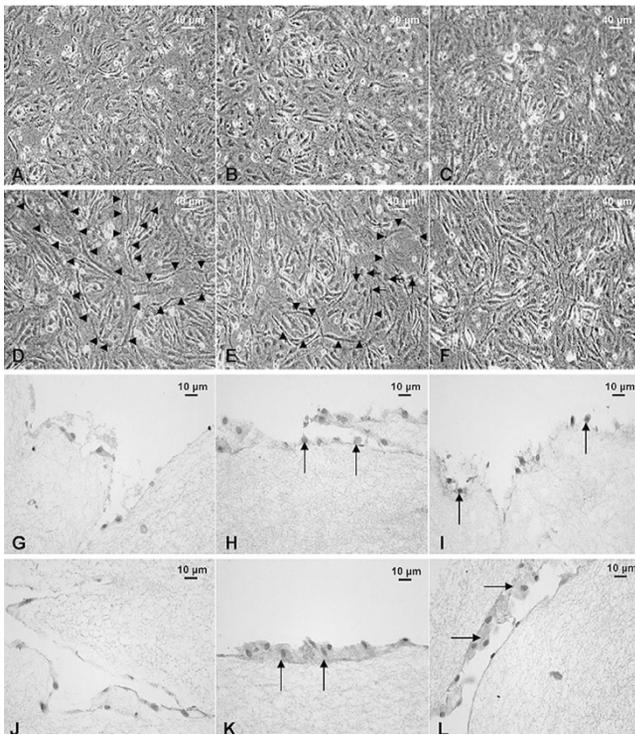
**3-OH-GA affects the integrity of endothelial tubes in vitro.** To determine whether 3-OH-GA affects the vascular morphogenesis, endothelial tube formation assays were performed. First, the tube formation was observed by phase contrast microscopy. Like the negative control (Fig. 2A), where collagen gels containing HDMEC were exposed to medium only, the treatment of gels with 1 (not shown), 2 (Fig. 2B), 4 (Fig. 2C), and 6 mM (not shown) 3-OH-GA did not induce any tube formation. VEGF (50 ng/mL) used as positive control induced an extensive capillary-like tube formation and network of tubes within 3–6 d (Fig. 2D, tubes marked by arrow heads). Simultaneous application of VEGF (50 ng/mL) and 3-OH-GA at 2 (Fig. 2E), 4 (Fig. 2F), and 6 mM (not shown) did not block formation of endothelial tubes but changed their morphologic appearance visible on the punctual interruption of tubes (marked by arrows) or tube network. Second, light microscopic evaluation of HE-stained paraffin sections obtained from gels shown in Figure 2, A–F, revealed that as the negative control (Fig. 2G), endothelial cells exposed to 3-OH-GA alone at 2 (Fig. 2H) and 4 mM (Fig. 2I) remained on top of the gel and did not form tubes. Whereas in VEGF-induced tubes endothelial cells were flattened and adherent



**Figure 1.** 3-OH-GA inhibits endothelial chemotaxis. (A) 3-OH-GA reduces the basal as well as VEGF-induced migration of HDMEC in a dose-dependent manner. The number of HDMEC migrating in the absence of effectors is indicated as control. Each bar represents the mean  $\pm$  SD of four independent experiments carried out in triplicates. \* $p < 0.05$ , \*\* $p < 0.01$  vs the respective control. (B) In direct comparison with 3-OH-GA at its most effective concentration, 6 mM GA and D2-OH-GA show no significant effects on cell migration whereas  $\alpha$ -KGA increased the number of migrated cells. VEGF (50 ng/mL) was used as positive control. Each bar represents the mean  $\pm$  SD of three experiments. \* $p < 0.05$ , \*\* $p < 0.01$  vs control.

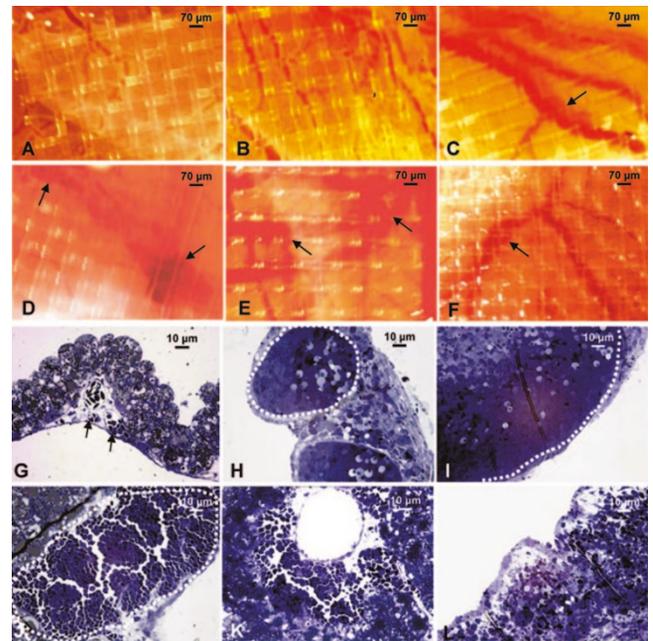
(Fig. 2J), the combined application of VEGF and 3-OH-GA at concentrations mentioned above led to roundly shaped and less adherent endothelial cells on top of the collagen gel (Fig. 2K) as well as within tubes (Fig. 2L) implicating an endothelial disorganization during capillary-like tube formation *in vitro*.

**3-OH-GA induces vascular dilatation and hemorrhage in CAM assay.** CAM assays were performed to study the role of 3-OH-GA on vascular morphogenesis *in vivo*. Compared with control (Fig. 3A), VEGF induced angiogenesis visible on the higher vascular density in the area of VEGF application (Fig. 3B). In contrast, treatment with 3-OH-GA alone at concentrations used in migration and tube formation assays did not lead to a higher vascularization but to abnormal dilatation of blood



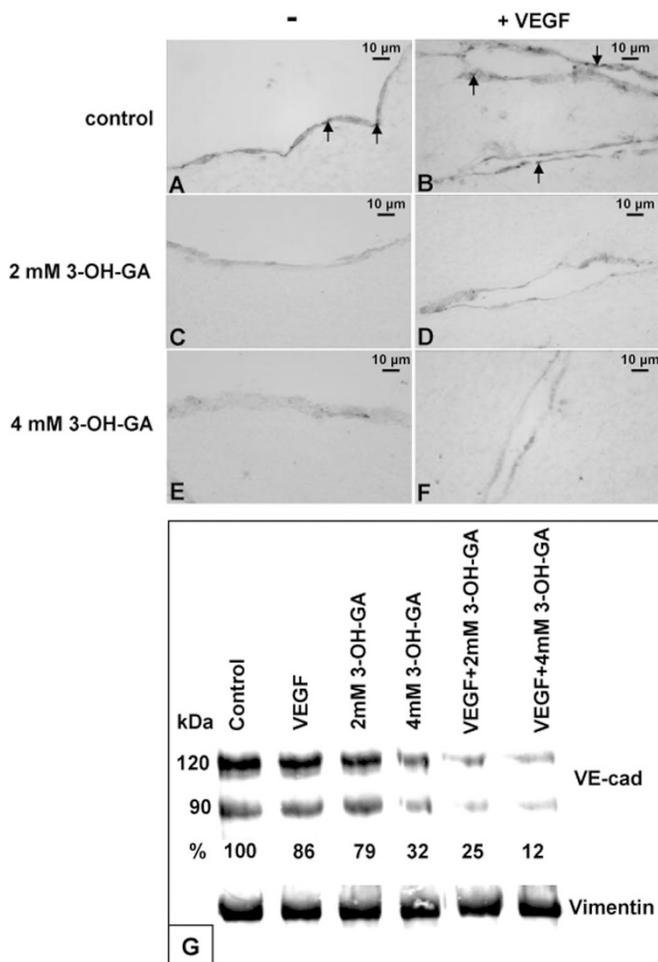
**Figure 2.** 3-OH-GA changes the morphology of VEGF-induced endothelial tubes. *A–F*: Phase contrast microscopy; *G–L*: light microscopic images of paraffin sections. Neither treatment of HDMEC with medium (*A*) nor 3-OH-GA-treatment at concentrations 2 (*B*) and 4 mM (*C*) alone induce endothelial tube formation, whereas VEGF (50 ng/mL) used as positive control (*D*) leads to formation of endothelial tubes with an extensive network (*arrow heads*). Simultaneous application of VEGF and 3-OH-GA at concentrations 2 (*E*) and 4 mM (*F*) does not alter the number of endothelial tubes but reduces the length and the network of endothelial tubes (*arrow heads*). The tubes are interrupted at several sites visible on the roundly shaped endothelial cells (*arrows*). Light microscopic evaluation of paraffin sections of HDMEC treated as demonstrated in *A–F* reveals that, in contrast to the negative control (*G*), 3-OH-GA-treated HDMEC (*H*, 2 mM, and *I*, 4 mM) are roundly shaped and in some areas less adherent to the underlying collagen gel (*arrows*). While HDMEC involved in endothelial tubes under VEGF treatment (*J*) are flattened as expected, those treated simultaneously with VEGF plus 2 (*K*) or 4 mM (*L*) 3-OH-GA are roundly shaped (*arrows*) on top of the collagen gel (not involved in tube formation) (*K*) as well as in several areas of VEGF-induced endothelial tubes (*L*), indicating a disintegration of endothelial cells during capillary morphogenesis.

vessels and to nonsharp vessel contours, implicating extravasation and hemorrhage, as shown for 4 mM 3-OH-GA (Fig. 3C). The vascular dilatation was enormous in areas of CAM blood vessels when 3-OH-GA was applied in combination with VEGF as shown for different 3-OH-GA concentrations (Fig. 3, *D–F*). Note that vessel contours were less sharp and hemorrhage was stronger than under 3-OH-GA alone. These findings were confirmed by light microscopic evaluation. Whereas blood vessels of CAM tissues treated with buffer exhibited a normal diameter (Fig. 3G), those treated with 3-OH-GA alone (Fig. 3H) or 3-OH-GA plus VEGF simultaneously (Fig. 3, *I–K*) were dilated dramatically and filled with blood cells completely. In some areas, an extensive extravasation of blood cells was observed (Fig. 3L).



**Figure 3.** 3-OH-GA leads to vascular dilatation and hemorrhage in CAM assay. In comparison to buffer control (*A*), VEGF-treatment leads to a considerable angiogenesis of CAM-tissue (*B*). 3-OH-GA-treatment alone (4 mM) results in vascular dilatation and decreased sharpness of vascular contours (*C*), presumably due to increased extravasation. Dramatic vascular dilatation and hemorrhage are observed when VEGF plus 2 (*D*), 4 (*E*), and 6 mM (*F*) 3-OH-GA are applied simultaneously. Light microscopic evaluation of semi-thin sectioned CAM tissues shows blood vessels (*arrows*) with normal diameter in buffer-treated areas (*G*). In contrast, vascular dilatation accompanied by stasis (blood vessel encircled by dotted line) is visible after treatment with 4 mM 3-OH-GA (*H*). In comparison to treatment with 3-OH-GA alone, diameter of CAM blood vessels is increased further when VEGF is applied with 2 (*I*), 4 (*J*), and 6 mM (*K*) 3-OH-GA. Additionally, the presence of 3-OH-GA leads to considerable extravasation of blood cells into CAM tissue as shown for VEGF plus 2 mM 3-OH-GA (*L*). Magnification (*G–L*)  $\times 350$ .

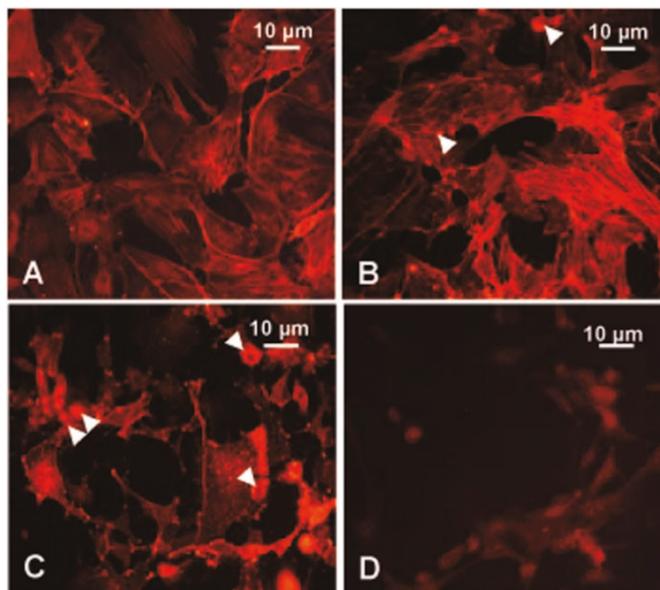
**3-OH-GA decreases the amount of VE-cadherin.** To elucidate the mechanisms of 3-OH-GA action on vascular morphogenesis and integrity, we studied the expression of different factors and cell adhesion molecules such as VEGF and VEGF receptors, VE-cadherin, and occludin by immunostaining. No changes were observed for VEGF, VEGF receptor 1 and 2, and occludin (data not shown). In contrast to HDMEC treated with medium (Fig. 4A) or VEGF alone (Fig. 4B), VE-cadherin immunostaining was reduced when 3-OH-GA was applied alone (Fig. 4, *C* and *E*) or in combination with VEGF (Fig. 4, *D* and *F*). Particularly, the accumulation at the contact zone between cells involved in endothelial tubes was decreased by 3-OH-GA. In a second approach, HDMEC growing in monolayer were incubated with 3-OH-GA alone (2 and 4 mM) and in combination with VEGF. Protein extracts of HDMEC were analyzed by Western blotting. These studies revealed that, regardless of the presence of VEGF, 3-OH-GA led to a significant reduction of the 120 kD-VE-cadherin band (Fig. 4G). A second band at 90 kD was detected, representing a degradation product of VE-cadherin (Fig. 4G).



**Figure 4.** 3-OH-GA reduces expression of VE-cadherin protein. VE-cadherin immunostaining was carried out on paraffin sections of *in vitro*-induced endothelial tubes as shown in Figure 2. VE-cadherin staining was observed in HDMEC incubated with medium (A, localized on the top of the gel) and in cells involved in the VEGF-induced tube formation (B). Note the particular accumulation of VE-cadherin at contact zones between HDMEC (arrows). In contrast, VE-cadherin immunostaining is reduced when HDMEC were treated with 3-OH-GA alone (C, 2 mM, and E, 4 mM) or simultaneously with VEGF plus 2 (D) or 4 mM (F) 3-OH-GA. Magnification (A–F)  $\times 350$ . VE-cadherin Western blot analysis (G) using extracts of monolayer-cultured HDMEC treated with VEGF and 3-OH-GA as indicated. Vimentin detection was used as loading control.

**3-OH-GA induces disassembly of actin cytoskeleton in HDMEC.** The effects of 3-OH-GA on endothelial migration and tube formation above allow one to assume that 3-OH-GA may affect the actin cytoskeleton of HDMEC. TRITC-phalloidin fluorescence staining of HDMEC revealed disassembly of actin cytoskeleton by application of 3-OH-GA (Fig. 5, B–D). In comparison to untreated HDMEC (Fig. 5A), punctual disorganization of actin cytoskeleton was already observed at 2 mM 3-OH-GA (Fig. 5B). Higher concentrations of 3-OH-GA (4 and 6 mM) resulted in extensive (Fig. 5C) until complete (Fig. 5D) disruption of actin stress fibers, respectively.

**3-OH-GA action on vascular endothelial cells is not mediated via NMDA receptors.** It has been proposed that cellular effects of 3-OH-GA on neuronal cells were mediated by



**Figure 5.** 3-OH-GA disrupts actin stress fibers in HDMEC. TRITC-phalloidin fluorescence staining of HDMEC demonstrates that 3-OH-GA treatment induces damage until complete disruption of actin cytoskeleton in a concentration-dependent manner. In contrast to organization of actin stress fibers in untreated HDMEC (A), 2 mM 3-OH-GA leads to punctual damage of actin fibers (B) visible on high number of red stained bulks (arrow heads), which are dominant in HDMEC treated with 4 mM 3-OH-GA (C). Finally, actin cytoskeleton is completely disrupted in the presence of 6 mM 3-OH-GA (D). Magnification:  $\times 450$ .

NMDA receptors. Analysis of mRNA expression of the constitutive NMDA receptor subunit 1 (NR1) by RT-PCR revealed that NR1 is expressed neither in treated nor in untreated HDMEC (not shown). These data suggest that in HDMEC the direct effects of 3-OH-GA are not mediated by NMDA receptors.

**DISCUSSION**

The present data show for the first time that 3-OH-GA affects structural integrity of blood vessels *via* disorganization of endothelial cells. These findings may explain vascular abnormalities such as hemorrhage and extravasation observed in subdural hematoma and acute retinal hemorrhage in patients with GA1 (2,16). We demonstrate here that 3-OH-GA, the disease-specific metabolite in GA1, impairs functional properties of HDMEC such as migration and morphogenesis of capillary-like tube formation *in vitro* in an NMDA receptor-independent manner, and induces abnormal dilatation of blood vessels accompanied by hemorrhage *in vivo*. Furthermore, 3-OH-GA treatment of HDMEC leads to disruption of actin cytoskeleton and reduces the amount of VE-cadherin in culture and during capillary-like tube formation *in vitro*.

The formation, patterning, and remodelling of the vascular system in basal and pathologic conditions are regulated by extracellular signals (17,18). These signals must be integrated by endothelial cells to control the formation of endothelial

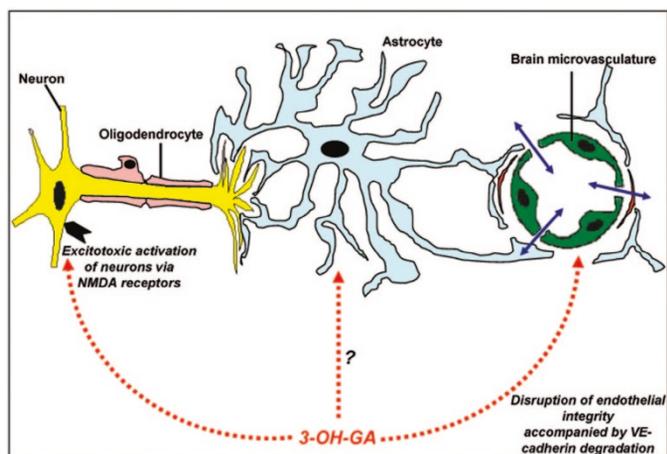
tubes as the first step of vascular morphogenesis. In this study, three experimental approaches were used to investigate direct effects of 3-OH-GA and underlying mechanisms on HDMEC. In the first *in vitro* model, 3-OH-GA inhibited both the basal as well as VEGF-induced migration of HDMEC in a concentration-dependent manner. The effect was specific for 3-OH-GA as GA, D2-OH-GA acid or  $\alpha$ -KGA rather increased than reduced the migration of endothelial cells. These data demonstrate a direct effect of 3-OH-GA on human primary vascular endothelial cells and implicate an inhibitory action of 3-OH-GA on a basic step of angiogenesis. However, these findings do not allow any further explanation on the physiologic significance regarding vessel morphology because this approach examines the chemotactic migration of isolated cells. The second *in vitro* assay provided evidence that 3-OH-GA disturbs the structural integrity of VEGF-induced endothelial tubes. Considering the fact that 3-OH-GA leads to a detachment of endothelial cells during VEGF-induced morphogenesis of capillary-like tubes *in vitro*, it seems to be paradox that 3-OH-GA also inhibits endothelial chemotaxis. However, our present data indicate that 3-OH-GA interferes at least with two distinct properties of endothelial cells: their motility and the interendothelial contacts as demonstrated by the increased degradation of VE-cadherin. Since 3-OH-GA disturbed also *in vivo* the integrity of both small and large blood vessels in CAM assay, it can be assumed that 3-OH-GA may affect basically the morphogenesis and maintenance of large and small blood vessels. In another model for brain injury in GA1, the intoxication with 3-nitropropionic acid (5), a dysfunction of the blood-brain-barrier endothelium has also been detected (19). Moreover, a loss of human cerebral endothelium barrier integrity has been observed in glutamate toxicity (20). Several studies have reported that actin-polymerization and the maintenance of microtubule dynamics are required for cell motility (21). Indeed, our findings show that 3-OH-GA interferes with the actin cytoskeleton, which may explain the effects of 3-OH-GA on endothelial cell motility.

The cell-cell adhesion molecules involved in the establishment of interendothelial junctions play a critical role for the formation and maintenance of blood vessels (22). Our study provides evidence that 3-OH-GA affects the expression of one of these proteins, VE-cadherin, in tube-formation experiments and in HDMEC grown in monolayer. VE-cadherin is the major cell-cell adhesion molecule at endothelial adherens junction (23). Its cytoplasmic domains contains binding sites for catenin p120 and plakoglobin, linking VE-cadherin to the actin and vimentin cytoskeleton network (24). The loss of VE-cadherin and plakoglobin results in abnormal assembly of endothelial cells into vascular structures during development, and disruption of endothelial cell-cell contacts, thus affecting barrier function (25,26). Because in 3-OH-GA-treated HDMEC we found no decrease of VE-Cadherin mRNA expression, it is likely that 3-OH-GA induces the degradation of VE-cadherin. Recent studies showed that VE-cadherin internalization followed by lysosomal/proteosomal degradation is an important regulatory mechanism controlling VE-cadherin cell surface expression (27). It remains to be examined

whether lysosomal/proteosomal inhibitors could prevent 3-OH-GA-induced reduction in VE-cadherin levels and stabilize capillary-like tubes.

Concentrations of 3-OH-GA required to affect endothelial cell properties are higher than those found *in vivo*. In circulation GA and 3-OH-GA have been detected at concentrations of 50  $\mu$ M and 30  $\mu$ M, respectively (1). However, in most studies examining *in vitro* effects of 3-OH-GA, *e.g.* on cell viability, growth factors, and reactive oxygen species, 3-OH-GA concentrations of 1–50 mM have been used for 24–72 h (28–32). In brain biopsy material of a GA1 patient, concentrations of GA and 3-OH-GA of 5 and 0.2 mM, respectively, have been reported (33). Especially a considerable rise of GA and 3-OH-GA during encephalopathic crises cannot be excluded, and so far there are no reports available concerning *in vivo* metabolite measurements during an encephalopathic crisis, instead of artifactual post mortem examinations. In brains of GCDH-deficient mice, GA and 3-OH-GA concentrations of 2 and 0.1 mM have been measured, respectively (34). Steady state concentrations of 3-OH-GA in the brain extracellular space, their regional differences as well as local changes during encephalopathic crises are unknown. Thus, a direct comparison between effective 3-OH-GA concentrations in patients and in *in vitro* cell models is difficult. Furthermore, it is considerable that the effects of 3-OH-GA on endothelial cells in concentrations of 4 mM as demonstrated here may also be observable when 3-OH-GA would be applied in lower concentrations but for a longer time period than we did here. Finally, it has been reported that human cerebral endothelial cells express NMDA receptors (20) and it is possible that cerebral endothelial cells response to lower concentrations of 3-OH-GA than HDMEC. Therefore, time course studies are needed to determine effects of 3-OH-GA on brain endothelial structures more precisely.

Taken together, the present data demonstrate that 3-OH-GA acts directly on vascular endothelial cells independently of NMDA receptors and affects the expression of VE-cadherin at the protein level. *In vitro* and *in vivo* studies show that 3-OH-GA leads to alterations of large and small blood vessels by endothelial disintegration leading to hemorrhage. These results may provide new insights into the pathophysiology of GA1 suggesting both NMDA receptor-dependent and -independent effects of 3-OH-GA on neuronal maintenance and vascular integrity (Fig. 6). The latter may explain the occurrence of vasogenic edema in GA1 patients or the formation of chronic subdural hemorrhages. At present, it is unclear whether the effects on vascular integrity are due to 3-OH-GA produced by brain cells, or plasma derived metabolites, or both. The used experimental approaches lack both the complexity of intracerebral cellular interactions and the developmental dynamics of the vascular system allowing only speculations on the selective damage of striatal blood vessels in GA1 patients. The susceptibility of these vessels to 3-OH-GA may be related to their developmental stage or the NMDA receptor expression level and might be affected by other brain cell-derived factors. Human striatal vessel development considerably differs from developing vasculature of other telencephalic



**Figure 6.** Dual effects of 3-OH-GA in cerebrovascular dysfunction. During acute encephalopathic crisis the 3-OH-GA concentration increases in the serum (circulation) and in the brain tissue leading to disruption of endothelial integrity due to elevated degradation of VE-cadherin. Hemorrhage and the exchange of 3-OH-GA as well as other metabolites and polypeptides between intra- and extravascular space may result in an alteration of brain extracellular microenvironment. This may additionally affect hemodynamics of brain blood vessels and neuronal cells *via* NMDA receptors, possibly leading to neuronal degeneration, particularly in the caudate and putamen. Disturbed endothelial integrity may contribute to the formation of local vasogenic rather than cytotoxic edema, further increasing the propensity of brain tissue to neurodegeneration. There are no data on whether 3-OH-GA affects astrocytes and the intercellular communication in the brain.

regions (35). This might explain region-specific vulnerability to 3-OH-GA in GA1. Subsequently, the intracranial hemodynamics as well as the local intrastriatal concentration of 3-OH-GA may be altered, further increasing the propensity for striatal injury. Like synaptogenesis and myelination, telencephalic angiogenesis is a dynamic process during the first 2 y of life, and coincides with the major window of neurologic vulnerability in children with GA1. Disturbed vascular biology during this time period may contribute to several hallmark features of the disease, including subdural and retinal hemorrhage, structural changes of the vascular wall and the peri-vascular spaces, and stroke-like putaminal necrosis.

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## REFERENCES

1. Goodman SI, Frerman FE 2001 Organic acidemias due to defects in lysine oxidation: 2-ketoadipic acidemia and glutaric acidemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B (eds) *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill Inc., New York, pp 2195–2204
2. Strauss KA, Puffenberger EG, Robinson DL, Morton DH 2003 Type I glutaric aciduria, part 1: natural history of 77 patients. *Am J Med Genet C Semin Med Genet* 121:38–52
3. Busquets C, Merinero B, Christensen E, Gelpi JL, Campistol J, Pineda M, Fernandez-Alvarez E, Prats JM, Sans A, Arteaga R, Marti M, Campos J, Martinez-Pardo M, Martinez-Bermejo A, Ruiz-Falco ML, Vaquerizo J, Orozco M, Ugarte M, Coll MJ, Ribes A 2000 Glutaryl-CoA dehydrogenase deficiency in Spain: evidence of two groups of patients, genetically, and biochemically distinct. *Pediatr Res* 48:315–322
4. Christensen E, Ribes A, Merinero B, Zschocke J 2004 Correlation of genotype and phenotype in glutaryl-CoA dehydrogenase deficiency. *J Inher Metab Dis* 27:861–868
5. Strauss KA, Morton DH 2003 Type I glutaric aciduria, part 2: a model of acute striatal necrosis. *Am J Med Genet C Semin Med Genet* 121:53–70
6. Kölker S, Koeller DM, Okun JG, Hoffmann GF 2004 Pathomechanisms of neurodegeneration in glutaryl-CoA dehydrogenase deficiency. *Ann Neurol* 55:7–12
7. Ullrich K, Flott-Rahmel B, Schluff P, Musshoff U, Das A, Lücke T, Steinfeld R, Christensen E, Jakobs C, Ludolph A, Neu A, Röper R 1999 Glutaric aciduria type I: pathomechanism of neurodegeneration. *J Inher Metab Dis* 22:392–403
8. Neumaier-Probst E, Harting I, Seitz A, Ding C, Kölker S 2004 Neuroradiological findings in glutaric aciduria type I (glutaryl-CoA dehydrogenase deficiency). *J Inher Metab Dis* 27:869–876
9. Funk CB, Prasad AN, Frosk P, Sauer S, Kölker S, Greenberg CR, Del Bigio MR 2005 Neuropathological, biochemical and molecular findings in a glutaric acidemia type I cohort. *Brain* 128:711–722
10. Ergün S, Kilic N, Ziegeler G, Hansen A, Nollau P, Götze J, Wurmbach JH, Horst A, Weil J, Fernando M, Wagener C 2000 CEA-related cell adhesion molecule 1: a potent angiogenic factor and a major effector of vascular endothelial growth factor. *Mol Cell* 5:311–320
11. Pepper MS, Ferrara N, Orci L, Montesano R 1992 Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis *in vitro*. *Biochem Biophys Res Commun* 189:824–831
12. Ergün S, Kilic N, Wurmbach JH, Ebrahimnejad A, Fernando M, Sevinc S, Kilic E, Chalajour F, Fiedler W, Lauke H, Lamszus K, Hammerer P, Weil J, Herbst H, Folkman J 2001 Endostatin inhibits angiogenesis by stabilization of newly formed endothelial tubes. *Angiogenesis* 4:193–206
13. Kilic N, Ergün S 2001 Methods to evaluate the formation and stabilization of blood vessels and their role in tumor growth and metastasis. In: Brooks SA, Schumacher U (eds) *Methods in Molecular Medicine, Vol. 58: Metastasis Research Protocols*, Vol. 2: Cell Behavior *In Vitro* and *In Vivo*. Humana Press Inc., Totowa, NJ, pp 125–148
14. Iruela-Arispe ML, Lane TF, Redmond D, Reilly M, Bolender RP, Kavanagh TJ, Sage EH 1995 Expression of SPARC during development of the chicken chorioallantoic membrane: evidence for regulated proteolysis *in vivo*. *Mol Biol Cell* 6:327–343
15. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294–5299
16. Hartley LM, Mrcp B, Khwaja OS, Verity CM, Frech B 2001 Glutaric aciduria type 1 and nonaccidental head injury. *Pediatrics* 107:174–175
17. Carmeliet P, Storkebaum E 2002 Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. *Semin Cell Dev Biol* 13:39–53
18. Folkman J 2003 Fundamental concepts of the angiogenic process. *Curr Mol Med* 3:643–651
19. Nishino H, Kumazaki M, Fukuda A, Fujimoto I, Shimano Y, Hida H, Sakurai T, Deshpande SB, Shimizu H, Morikawa S, Inubushi T 1997 Acute 3-nitropropionic acid intoxication induces striatal astrocytic cell death and dysfunction of the blood-brain barrier: involvement of dopamine toxicity. *Neurosci Res* 27:343–355
20. Sharp CD, Hines I, Houghton J, Warren A, Jackson TH 4th, Jawahar A, Nanda A, Elrod JW, Long A, Minagar A, Alexander JS 2003 Glutamate causes a loss in human cerebral endothelial barrier integrity through the activation of the NMDA receptor. *Am J Physiol Heart Circ Physiol* 285:H2592–2598
21. Pollard TD, Blanchoin L, Mullins RD 2000 Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu Rev Biophys Biomol Struct* 29:545–576
22. Dejana E 1997 Endothelial adherens junctions: implications in the control of vascular permeability and angiogenesis. *J Clin Invest* 100:S7–S10
23. Breviaro F, Caveda L, Corada M, Martin-Padura I, Navarro P, Golay J, Introna M, Gulino D, Lampugnani MG, Dejana E 1995 Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin. *Arterioscler Thromb Vasc Biol* 15:1229–1239
24. Angst BD, Marozzi C, Magee AI 2001 The cadherin superfamily: diversity in form and function. *J Cell Sci* 114:629–641
25. Schnittler HJ, Puschel B, Drenckhahn D 1997 Role of cadherins and plakoglobin in interendothelial adhesion under resting conditions and shear stress. *Am J Physiol* 273:H2396–2405
26. Vittel D, Buchou T, Schweitzer A, Dejana E, Huber P 1997 Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc Natl Acad Sci U S A* 94:6273–6278
27. Xiao K, Allison DF, Kottke MD, Summers S, Sorescu GP, Faundez V, Kowalczyk AP 2003 Mechanisms of VE-cadherin processing and degradation in microvascular endothelial cells. *J Biol Chem* 278:19199–19208
28. Bjugstad KB, Zawada WM, Goodman SI, Freed CR 2001 IGF-1 and bFGF reduced glutaric acid and 3-hydroxyglutaric acid toxicity in striatal cultures. *J Inher Metab Dis* 24:631–647
29. Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF 1999 3-Hydroxyglutaric acid

- glutaric acids are neurotoxic through NMDA receptors *in vitro*. *J Inher Metab Dis* 22:259–262
30. Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF 2001 Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons. *Pediatr Res* 50:76–82
  31. Kölker S, Köhr G, Ahlemeyer B, Okun JG, Pawlak V, Hörster F, Mayatepek E, Krieglstein J, Hoffmann GF 2002  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons. *Pediatr Res* 52:199–206
  32. Latini A, Borba Rosa R, Scussiato K, Llesuy S, Bello-Klein A, Wajner M 2002 3-hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. *Brain Res* 956:367–373
  33. Külkens S, Harting I, Sauer S, Zschocke J, Hoffmann GF, Gruber S, Bodamer OA, Kölker S 2005 Late-onset neurologic disease in glutaryl-CoA dehydrogenase deficiency. *Neurology* 64:2142–2144
  34. Koeller DM, Woontner M, Crnic LS, Kleinschmidt-DeMasters B, Stephens J, Hunt EL, Goodman SI 2002 Biochemical, pathologic and behavioral analysis of a mouse model of glutaric acidemia type I. *Hum Mol Genet* 11:347–357
  35. Kuban KC, Gilles FH 1985 Human telencephalic angiogenesis. *Ann Neurol* 17:539–548