β-Cell-protective effect of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid as a glutamate dehydrogenase activator in db/db mice

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Abstract

2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) is an activator of glutamate dehydrogenase (GDH), which is a mitochondrial enzyme with an important role in insulin secretion. We investigated the effect of BCH on the high-glucose (HG)-induced reduction in glucose-stimulated insulin secretion (GSIS), the HG/palmitate (PA)-induced reduction in insulin gene expression, and HG/PA-induced β-cell death. We also studied whether long-term treatment with BCH lowers blood glucose and improves β-cell integrity in db/db mice. We evaluated GSIS, insulin gene expression, and DNA fragmentation in INS-1 cells exposed to HG or HG/PA in the presence or absence of BCH. An in vivo study was performed in which 7-week-old diabetic db/db mice were treated with BCH (0.7 g/kg, n = 10) and placebo (n = 10) every other day for 6 weeks. After treatment, an intraperitoneal glucose tolerance test and immunohistological examinations were performed. Treatment with BCH blocked HG-induced GSIS inhibition and the HG/PA-induced reduction in insulin gene expression in INS-1 cells. In addition, BCH significantly reduced HG/PA-induced insulin cell death and phospho-JNK level. BCH treatment improved glucose tolerance and insulin secretion in db/db mice. BCH treatment also increased the ratio of insulin-positive β-cells to total islet area (P < 0.05) and reduced the percentage of β-cells expressing cleaved caspase 3 (P < 0.05). In conclusion, the GDH activator BCH improved glycemic control in db/db mice. This anti-diabetic effect may be associated with improved insulin secretion, preserved islet architecture, and reduced β-cell apoptosis.


Introduction

Pancreatic β-cells produce the hormone insulin, whose action on target tissues maintains glucose homeostasis. Mitochondria play a pivotal role by generating signals that couple glucose sensing to insulin secretion (Maechler & Wollheim 2001, Frigerio et al. 2008). The mitochondrial matrix enzyme glutamate dehydrogenase (GDH), which catalyzes the interconversion of glutamate and the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate, is an important enzyme in the regulation of insulin secretion in pancreatic β-cells (Hudson & Daniel 1993). The function of GDH as the regulator of insulin secretion was established through studies on the insulin-releasing capacity of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH; Sener & Malaisse 1980, Sener et al. 1981). These studies showed that BCH increased insulin secretion by activating GDH and that the insulino-tropic action of BCH correlated with its ability to increase the oxidative deamination of glutamate. The discovery that gain-of-function mutations in the gene encoding GDH cause hyperinsulinism/hyperammonemia syndrome underlined the importance of GDH in glucose homeostasis in humans (Stanley et al. 1998, 2000, Tanizawa et al. 2002).

The activation of GDH by allosteric activators such as leucine and BCH as well as the overexpression of constitutively activated GDH induce insulin secretion through enhanced glutamate oxidation (Sener et al. 1981, Panten et al. 1984, Gao et al. 1999, Anno et al. 2004, Carobbio et al. 2004). It is, therefore, generally accepted that the activation of GDH enhances oxidative deamination of glutamate and increases ATP production by supplying mitochondria with α-ketoglutarate, thereby stimulating insulin secretion (Sener & Malaisse 1980, Sener et al. 1981, Kelly et al. 2002, Anno et al. 2004).

Based on the results of previous studies, glucose concentration appears to be altered in GDH-overexpressing islets in a way that increases glucose-stimulated insulin secretion (GSIS; Anno et al. 2004, Carobbio et al. 2004).
One study reported that at low-glucose (LG) concentration, GDH overexpression in islets potentiated insulin secretion (Anno et al. 2004). However, another study showed that this was potentiated at high-glucose (HG) concentration (Carobbio et al. 2004). First- and second-phase insulin secretion was impaired in β-cell-specific GDH deletion mice, indicating that GDH-dependent amplification of the secretory response may affect both phases (Carobbio et al. 2009). It was noted in a recent study that by ADP-ribosylating GDH, SIRT4 repressed the activity of GDH, thereby downregulating insulin secretion in pancreatic β-cells (Haigis et al. 2006).

Recently, we demonstrated that metabolic impairment in mitochondria is associated with β-cell glucolipotoxicity (Choi et al. 2011). According to these results, long-term treatment of INS-1 cells with HG/palmitate (PA) impaired energy-producing metabolism and caused depletion of TCA cycle intermediates. Treatment with BCH not only maintained the TCA cycle intermediate pool but also had a strong protective effect against HG/PA-induced β-cell death. Therefore, we initially examined whether BCH improved β-cell glucolipotoxicity, as measured by HG-induced reduction in GSIS, HG/PA-induced reduction in insulin gene expression, and HG/PA-induced β-cell death. Next, we investigated which death-related signal is affected by BCH. We also studied the effects of BCH on glycemic control and pancreatic β-cell integrity in db/db mice.

Materials and Methods

Cells

INS-1 rat insulinoma cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, 100 g/ml streptomycin, and 10 mM HEPES at 37 °C in a humidified atmosphere of 95% air and 5% CO2.

Preparation of PA

PA/BSA conjugates were prepared as described previously (Choi et al. 2011). Briefly, a 20 mM solution of PA in 0-01 M NaOH was incubated at 70 °C for 30 min and the fatty acid soaps were then complexed with 5% fatty acid-free BSA in PBS in 1:3 volume ratio. The complexed fatty acids consisted of 5 mM PA and 3-75% BSA. The PA/BSA conjugates were diluted in 10% FBS culture medium (~0-4% BSA) and administered to cultured cells.

Measurement of insulin secretion and content

INS-1 cells (2X10^5) growing in 24-well plates were washed twice with KRB buffer (24 mM NaHCO2, 1:2 mM MgCl2, 1 mM HEPES, 129 mM NaCl, 4:8 mM KCl, 1:2 mM KH2PO4, 2:5 mM CaCl2, 0:2% BSA, and 0:2 mM glucose, pH 7-4) and then incubated in the same buffer for 1 h. Insulin secretion was stimulated by treatment with 0:2 or 16:7 mM glucose for 2 h. At the end of the incubation period, the amount of insulin released into the supernatant was quantified using a rat insulin RIA kit (Linco Research, St Charles, MO, USA).

To determine insulin content, the cells were washed twice with KRB buffer and incubated overnight in ethanol/HCl buffer at 4 °C. At the end of the incubation period, the supernatant was collected and subjected to rat insulin RIA.

Apoptosis ELISA assay

Cells seeded in 96-well plates were grown in RPMI 1640 containing 11-1 mM glucose until they reached 70% confluence. They were then treated with 25 mM HG and 0:4 mM PA in the presence or absence of BCH for 24 h. The cells were lysed, and cytosolic levels of oligonucleosomes, produced as a result of apoptosis-related DNA degradation, were quantified using a Cell Death Detection ELISA Plus kit (Roche Applied Science) according to the manufacturer’s instructions.

Quantitative real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed using 1 μg total RNA and an avian myeloblastosis virus reverse transcription system. PCR was performed using a SYBR Green PCR Master Mix (PE Applied Biosystems, www.appliedbiosciences.com) according to the manufacturer’s instructions. The following primers were used: insulin 1, CTGGTGAGGCCTCTGTACCT (forward) and GTGCAGCAGTGTCCAACAT (reverse) and GAPDH, ATGATTCTACCCAGGAAG (forward) and CTGGAAATGTTGATGGTT (reverse). All amplification was performed using the following thermal cycling conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The real-time PCR analysis was performed using an Applied Biosystems Prism 7900 Sequence Detection System (PE Applied Biosystems). Expression data were normalized to the values for the control gene GAPDH to yield the relative abundance.

Immunoblotting

Whole proteins were extracted by differential centrifugation (10 000 g, 10 min) and protein concentrations in lysates were determined using protein assay kits (Bio-Rad). An equal volume of 2X SDS sample buffer (125 mM Tris–Cl (pH 6:8), 4% SDS, 4% 2-mercaptoethanol, 20% glycerol) was added to cell lysates, and equivalent amounts of protein (30 μg) were loaded onto 10–15% polyacrylamide gels, electrophoresed, and then electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA).


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After blocking the membranes with 5% skimmed milk for 30 min, target antigens were reacted with anti-phospho-JNK (Thr183/Tyr185; Cell Signaling, Beverly, MA, USA), anti-total JNK, or anti-β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as primary antibody and then with the appropriate secondary antibody: HRP-conjugated anti-rabbit IgG for anti-total JNK and anti-phospho-JNK antibodies or anti-goat IgG for anti-β-actin antibody. Immunoreactive bands were then developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Band intensity was determined by densitometric analysis using a one-dimensional Quantity One 1D image analysis system.

**Animals and treatments**

Five-week-old male diabetic db/db (C57BLKS/J-leprdb/leprdb) mice and male nondiabetic db/+ (C57BLKS/J-leprdb/+) mice were purchased from Japan SLC (Shizuoka, Japan). The animals were housed three per cage in a temperature-controlled room (22°C) under a 12 h light:12 h darkness cycle with lights on at 0600 h. The animals were given free access to a standard chow diet and water. Following 2 weeks of adaptation, the mice were randomly divided into three groups (n=10/group): saline-injected nondiabetic db/+ mice, saline-injected diabetic db/db mice (db/db control mice), and BCH-injected diabetic db/db mice. Before use, BCH (Sigma) was dissolved in saline to yield a 10 mM stock solution. BCH (0.7 g/kg) was injected intraperitoneally every other day for 6 weeks. All institutional guidelines for the care and use of animals were followed.

**Intraperitoneal glucose tolerance test**

After 6 weeks of treatment, the mice were fasted for 16 h before the intraperitoneal glucose tolerance test (IPGTT), to allow complete drug washout. Glucose (2 g/kg) was administered via injection into the peritoneal cavity. Blood samples were collected from tails using heparinized calibrated micropipettes at 0, 60, and 180 min after glucose loading. Blood was immediately centrifuged at 400 g for 10 min at 4°C, and the plasma was collected and stored at −80°C until assayed. Plasma glucose levels were measured using the glucose oxidase method, and plasma insulin levels were determined using an RIA kit (Linco Research).

**Histology, immunohistochemistry, and immunofluorescence**

At 14 weeks of age, the mice were killed. The pancreas of each mouse was removed, fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned to a thickness of 3 μm. Pancreatic sections were stained first with hematoxylin and eosin (H&E) and then for insulin (with a guinea pig anti-insulin antibody; Dako Corp., Carpinteria, CA, USA) and glucagon (rabbit anti-glucagon antibody; Cell Signaling Technology). Additional sections were co-stained with fluorescently labeled antibodies specific for insulin (Dako Corp.) and cleaved caspase 3 (Cell Signaling Technology). Fluorescence signals were visualized using a confocal microscope (Carl Zeiss MicroImaging GmbH), with excitation at 448 nm and emission at 515 nm. Finally, numbers of cleaved caspase 3-positive β-cells in each stained section were counted at ×400 magnification. The frequency of cleaved caspase 3-positive β-cells was determined as a percentage of the total number of β-cells in each tissue section. Histological images were analyzed using ImageJ software (NIH Image, Bethesda, MA, USA).

**Statistical analyses**

All data are expressed as mean ± S.E.M. Statistically significant differences were identified using Student’s t-test (for assays with two sample sets) and one-way ANOVA in conjunction with the Tukey correction (for multiple experimental groups). Statistical analyses were performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). A P value of <0.05 was deemed to indicate statistical significance.

**Results**

**Potentiating effect of BCH on insulin secretion in INS-1 cells**

Insulin secretion by INS-1 cells was stimulated by treatment with 0-2 mM glucose (LG) or 16-7 mM glucose (HG) for 2 h, in conjunction with 10 mM BCH. As shown in Fig. 1A, HG increased insulin secretion about 2-0-fold compared with LG. BCH augmented LG- and HG-stimulated insulin secretion by approximately 1-8- and 1-2-fold respectively. Thus, BCH potentiated insulin secretion stimulated by LG and HG in INS-1 cells.

**Preventive effect of BCH on HG-induced GSIS inhibition and HG-induced reduction of insulin content in INS-1 cells**

To determine whether treatment with BCH can prevent HG-induced GSIS inhibition in β-cells, INS-1 cells were incubated with BCH in medium containing 5-6 or 25 mM glucose for 24 h, and the amount of insulin secreted in response to glucose stimulation (LG: 0-2 mM or HG: 16-7 mM) for 2 h was measured by RIA. As shown in Fig. 1B, GSIS in INS-1 cells was impaired by long-term exposure to HG, whereas treatment with BCH significantly prevented HG-induced GSIS inhibition. Treatment with BCH did not affect LG-induced insulin secretion.

In addition, while long-term incubation with HG significantly reduced insulin content, treatment with BCH significantly restored HG-induced reduction of insulin content in a concentration-dependent manner (Fig. 1C).
Protective effect of BCH on the HG/PA-induced reduction in insulin gene expression in INS-1 cells

The exposure of INS-1 cells to 25 mM glucose and 0.4 mM PA (HG/PA) for 12 h reduced insulin gene expression by 60% compared with BSA control treatment (Fig. 1D). Co-treatment with BCH blocked the inhibitory effect of HG/PA on insulin gene expression in a concentration-dependent manner.

Protective effect of BCH against HG/PA-induced INS-1 cell death

A cell death detection assay revealed fragmented DNA in INS-1 cells exposed to HG/PA for 24 h (Fig. 2A). HG/PA increased cytosolic DNA fragmentation from 0.3 to 1.6, indicating that it induced apoptotic cell death in INS-1 cells. BCH protected against HG/PA-induced DNA fragmentation in a concentration-dependent manner. As JNK is known to be a critical mediator in HG/PA-induced ß-cell apoptosis, we investigated whether JNK activation was associated with the protective effect of BCH. Treatment with BCH attenuated HG/PA-induced phospho-JNK level in a concentration-dependent manner (Fig. 2B and C).

Effect of BCH treatment on body weight, plasma glucose level, and plasma insulin level during IPGTT

In the preliminary experiments, we injected 7-week-old db/db mice (n = 6 per group) with BCH at a dose of 0.35 g/kg (2.26 mmol/kg) or 0.7 g/kg (4.5 mmol/kg) every other day for 4 weeks. We found that 0.7 g/kg BCH was more effective
than 0.35 g/kg BCH for lowering the glucose level. We thus decided to use BCH at a dose of 0.7 g/kg in our study but extended the treatment time from 4 to 6 weeks (Supplementary Figure 1, see section on supplementary data given at the end of this article).

After 6 weeks of treatment, body weight was significantly higher in db/db control mice (43.6 ± 2.5 g) than in nondiabetic db/+ mice (37.2 ± 0.8 g). However, there was no difference in body weight between db/db control mice and db/db mice treated with BCH (44.5 ± 2.2 g). As shown in Fig. 3A, db/db control mice exhibited marked glucose intolerance, as confirmed by the results of an IPGTT. Interestingly, BCH treatment significantly improved glucose tolerance and insulin secretion (Fig. 3A and B).

Effect of BCH treatment on islet morphology

Pancreatic tissue from db/db control mice showed islet destruction and decreased β-cell insulin staining compared with db/+ mice (Fig. 4A). In addition, islets from db/db control mice contained glucagon-positive α-cells, which had infiltrated the entire islet. Tissue from BCH-treated db/db mice displayed relatively normal islet morphology, significantly reduced number of α-cells in the islet core, and a more normal distribution of β-cells and α-cells. The ratios of insulin-positive β-cells to total islet area and glucagon-positive α-cells to total islet area were calculated from acquired digital images. As shown in Fig. 4B, the insulin-positive β-cells accounted for 79.2 ± 1.2% of the total islet area in nondiabetic db/+ mice, but only 42.2 ± 1.7% in db/db control mice (P < 0.05). Treatment with BCH significantly increased the insulin-positive β-cell-to-total islet area ratio (56.0 ± 0.8%, P < 0.05; Fig. 4C). On the other hand, the glucagon-positive α-cell-to-total islet area ratio was higher in db/db control mice than in nondiabetic db/+ mice (16.6 ± 2.2 vs 10.3 ± 0.8%, P < 0.05; Fig. 4C). Treatment with BCH significantly reduced the glucagon-positive α-cell-to-total islet area ratio (11.2 ± 0.5%).

Effect of BCH treatment on cleaved caspase 3 expression in β-cells

We performed immunofluorescence analyses to determine whether the expression of cleaved caspase 3 in β-cells was altered in animals treated with BCH. The expression of cleaved caspase 3 was significantly lower in BCH-treated db/db mice compared with db/db control mice (5.4 ± 0.9 vs 15.6 ± 2.8, P < 0.05; Fig. 5A and B).

Discussion

We have known for a while that BCH induces hypoglycemia and stimulates insulin release (Christensen & Cullen 1969, Christensen et al. 1971, Fajans et al. 1971). Later, it was shown that the sole mechanism of action of BCH is the activation of GDH (Sener & Malaisse 1980, Sener et al. 1981). Until now,
most studies on BCH as a GDH activator have focused on its function in insulin secretion, and not any other functions in β-cells.

In this study, we found that BCH reversed HG-induced GSIS inhibition and protected against HG/PA-induced reduction of insulin gene expression as well as HG/PA-induced β-cell cytotoxicity in vitro. Furthermore, in accordance with its protective effect against HG/PA-induced glucolipotoxicity, BCH preserved β-cell mass and improved glycemic control in db/db mice. To our knowledge, this is the first report demonstrating the protective effects of BCH on β-cell function and islet architecture in a type 2 diabetes animal model.

Chronic exposure to elevated levels of glucose and fatty acids is thought to contribute to the progression of type 2 diabetes by impairing β-cell function and inducing β-cell apoptosis, effects that are collectively referred to as β-cell glucolipotoxicity (El-Assaad et al. 2003, 2010, Poitout & Robertson 2008). Mitochondria mediate the response of β-cells to extracellular glucose by generating ATP and initiating a cascade of events culminating in the release of insulin. However, β-cells chronically exposed to high levels of glucose and free fatty acids showed reductions in mitochondrial membrane potential and ATP production (El-Assaad et al. 2010). Furthermore, decreased levels of various mitochondrial enzymes in islet cells under hyperglycemic and hyperlipidemic conditions support the theory that mitochondrial dysfunction contributes to β-cell glucolipotoxicity (MacDonald et al. 2009, Lu et al. 2010).

We hypothesized that the anti-diabetic effect of BCH results from improved mitochondrial function owing to the activation of GDH. The first contributor to this effect of BCH is the stimulation of insulin secretion. Islets acutely exposed to HG levels showed an increase in insulin release, whereas a decrease in insulin release was observed in islets exposed chronically to HG (Eizirik et al. 1992, Marshak et al. 1999). Our results showed that acute treatment with BCH potentiated insulin secretion and that long-term treatment blocked HG-induced GSIS inhibition in INS-1 cells and increased insulin secretion in db/db mice. These results are in...
BCH is protection against HG/PA-induced β-cell apoptosis. Based on these results, we concluded that BCH improved insulin gene expression under glucolipotoxic conditions in db/db vs control mice. In our previous study, when the HG/PA-induced reduction in viability began to be detected, TCA cycle intermediates, restored ATP levels, enhanced the oxidation rate, and protected against HG/PA-induced INS-1 cell apoptosis.

The JNK pathway is known to be a critical mediator of fatty acid-induced endoplasmic reticulum (ER) stress (Martinez et al. 2008). Bachar et al. (2009) showed that glucose increased JNK phosphorylation in response to PA-induced ER stress and that JNK inhibition reduced β-cell apoptosis, underlining the importance of this pathway in mediating glucolipotoxicity-induced β-cell apoptosis. In this study, BCH suppressed HG/PA-induced JNK activation in INS-1 cells. We also confirmed the protective effects of BCH against β-cell apoptosis in db/db mice. BCH protected against loss of β-cell mass and preserved the architecture of pancreatic islets. Another mechanism may be the reduction in β-cell overwork due to insulin resistance, as BCH treatment improved insulin sensitivity in insulin tolerance tests in db/db mice (data not shown). We, therefore, cannot exclude the possibility that the anti-diabetic effect of BCH was attributable to enhanced glycemic control and that BCH thus indirectly reduced glucolipotoxicity.

Finally, we suggest that the activation of GDH by BCH may enhance mitochondrial energy metabolism by increasing anaplerotic flux, thereby improving β-cell function and preventing β-cell apoptosis.

In a recent study, transgenic mice with β-cell-specific deletion of GDH exhibited a 60% reduction in GDH activity, an increased percentage of disorganized islets, and a partial reduction in GSIS (Carobbio et al. 2009). These results are consistent with our findings that GDH is important in insulin secretion and islet integrity. However, when they were fed a normal-calorie diet, these transgenic mice did not show a difference in IPGTT glucose excursion compared with control mice. Because of this, the authors suggested that although insulin secretion was strongly limited, maximal GDH capacity is not required to maintain glucose homeostasis under normal-calorie conditions. Thus, we believe that if these transgenic mice with β-cell-specific deletion of GDH were fed other high-nutrient diets such as HG or high-free fatty acid diets, they would develop glucose intolerance.

Treatment with BCH was not lethal and did not affect body weight, weights of major organs, food intake, or physical appearance (Supplementary Table 1, see section on supplementary data given at the end of this article). We did not detect any abnormalities upon autopsy of BCH-treated db/db mice. Moreover, treatment with BCH did not cause liver toxicity, as assessed by serum aspartate aminotransferase and alanine aminotransferase levels (Supplementary Table 1). These findings suggest that the dose of BCH used did not cause any toxicity in db/db mice.

The dose of BCH used in this study and another study (Christensen & Cullen 1969) may be too high for therapeutic treatment of diabetes, and lower doses of BCH could be used for future studies.

Figure 5 Effect of BCH on β-cell expression of cleaved caspase 3, as assessed by immunofluorescence. (A) Representative images of islets stained for insulin (red) and cleaved caspase 3 (green). (B) Cleaved caspase 3-positive β-cell frequencies. Tissue sections from saline-treated db/+ mice and db/db mice treated with saline (db/db control) or BCH (db/db BCH) were analyzed. Data are expressed as mean±s.e.m. (n=6 per group). *P<0.05 vs db/+ mice; †P<0.05 vs db/db control mice.
usefulness in the treatment of type 2 diabetes. A new chemical that can effectively mimic BCH at lower dosages (i.e., μmol amounts) is needed. In addition, the effect of BCH can be influenced by the supply of glutamate.

In summary, we demonstrated that treatment with the GDH activator BCH improved glucose tolerance in db/db mice, a type 2 diabetes animal model. Improved glycemic control was associated with improved insulin secretion, increased insulin-positive β-cell area as a percentage of total islet area, restoration of normal islet architecture, and reduced β-cell apoptosis. The results suggest that pharmacological activation of GDH may represent a novel therapy for type 2 diabetes.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-11-0340.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This study was supported by a new faculty research fund at Ajou University School of Medicine (2009) and by grants from the Seisun Research Fund (2009); KRF (grant number 2009-0072584, 2011-0022041); the GRRC Program of Gyeonggi Province (grant number GRRCJOU2011-B02); Daewoong; MSD Korea; and the Korean Health Technology R&D Project, Program of Gyeonggi Province (grant number GRRCAJOU2011-B02); KRF (grant number 2009-0072584, 2011-0022041); the GRRC School of Medicine (2009) and by grants from the Seisun Research Fund (2009-0072584, 2011-0022041).

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Received in final form 10 November 2011
Accepted 30 November 2011
Made available online as an Accepted Preprint 30 November 2011