Hypothalamic reactive oxygen species are required for insulin-induced food intake inhibition. A NADPH oxidase-dependent mechanism.

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Objective: Insulin plays an important role in the hypothalamic control of energy balance, especially by reducing food intake. Emerging data points out a pivotal role of reactive oxygen species (ROS) in the energy homeostasis regulation, but their involvement in the anorexigenic effect of insulin is unknown. Furthermore, ROS signal derived from NADPH oxidase activation is required for physiological insulin effects in peripheral cells. In this study, we investigated the involvement of hypothalamic ROS and NADPH oxidase in the feeding behavior regulation by insulin.

Research design and methods: We first measured hypothalamic ROS level and food intake after acute intracerebroventricular (icv) injection of insulin. Second, effect of a pretreatment with a ROS scavenger or a NADPH oxidase inhibitor was evaluated. Third, we examined the consequences of two nutritional conditions of central insulin unresponsiveness (fasting or short-term high-fat diet) on the insulin ability to modify ROS level and food intake.

Results: In normal chow-fed mice, insulin inhibited food intake. At the same dose, insulin rapidly and transiently increased hypothalamic ROS level by 36%. The pharmacological suppression of this insulin-stimulated ROS elevation, either by antioxidant or by a NADPH oxidase inhibitor abolished the anorexigenic effect of insulin. Finally, in fasted- and short-term high-fat diet-fed mice insulin did not promote any more elevation of ROS level and food intake inhibition, likely due to higher hypothalamic diet-induced antioxidant defence system.

Conclusions: A hypothalamic ROS increase through NADPH oxidase is required for the anorexigenic effect of insulin.
The hypothalamus is a cerebral area involved in the regulation of energy homeostasis. In this context, insulin plays a pivotal role. By acting in the hypothalamus, insulin reduces food intake and body weight (1-2), activates sympathetic nerve outflow to the brown adipose tissue (3) and suppresses hepatic endogenous glucose production (4-5). In situation of excess nutrient intake the hypothalamus rapidly becomes resistant to insulin before obesity and diabetes onset (6-7). Moreover, inactivation of the neuronal insulin receptor leads to the development of diet-induced obesity with an increase in body fat, a mild insulin resistance and elevated plasma insulin levels (8). Therefore, it is of prime importance to elucidate cellular mechanisms by which insulin acts on hypothalamic cells to understand central early-onset diet-induced obesity and diabetes.

In recent years, emerging data points out a pivotal role of reactive oxygen species (ROS) in the energy homeostasis regulation by the hypothalamus. In response to an acute overload of nutrients, a subtle rise of the ROS concentration within this area is sufficient to reduce food intake (9) or to stimulate parasympathetic nervous system and pancreatic insulin secretion (10). Furthermore, the gut-derived hormone ghrelin exerts its central effect on feeding behavior by controlling hypothalamic ROS levels (11). However, the role of ROS in the anorexigenic effect of insulin and more generally in the brain insulin signaling remains unknown. In peripheral insulin-sensitive cells, ROS have been shown to control the crucial early steps in insulin signaling (12-14). Moreover, they contribute to the propagation of the insulin cascade in these cells (15-17). Thus, transient bursts of small amounts of ROS triggered in response to insulin facilitate both early and distal insulin signaling pathway. Reinforcing this concept, it has been recently shown that this transient insulin-induced ROS production is also required in neuronal cells for the enhancement of insulin receptor autophosphorylation, the first step in insulin signaling pathway (18). These data lead us to hypothesize that insulin might trigger ROS elevation within the hypothalamus, which in turn, would inhibit food intake.

In peripheral cells, it is well established that both insulin-induced ROS rise and insulin cascade activation are dependent of NADPH oxidase activity (12-17, 19). Blocking the insulin-induced ROS production with an inhibitor of NADPH oxidase activity (diphenyleneiodonium) dramatically reduces insulin signaling pathway activation and thus its physiological effects (15).

Despite the demonstration of the crucial role of ROS signaling and NADPH oxidase in peripheral insulin effects, their involvement in the brain insulin signaling has not yet been investigated. Therefore, in this study we tested whether the anorexigenic insulin effect required a ROS-dependent signaling pathway within the hypothalamus. For this purpose, we measured hypothalamic ROS level and food intake after intracerebroventricular insulin injection. The involvement of ROS and NADPH oxidase in the insulin-induced food intake inhibition was evaluated by pretreatment with a ROS scavenger or a NADPH oxidase inhibitor. Since central insulin responsiveness is known to be altered by a short-term deficit (20) as well as an excess (7) of nutrients availability, we examined the consequence of 18 hours fasting or 3 days high-fat diet on the insulin ability to modify hypothalamic ROS level and food intake.

MATERIALS AND METHODS

Animals. All animal experiments were carried out in strict accordance with the
European Communities Council directive (86/609/EEC) and the practical guide for the care and use of laboratory animals edited by the National Research Council. For this study, we used 7- to 8-weeks-old male C57BL6/J mice (Harlan laboratories). The animals were kept in a temperature-controlled room (22 ± 1°C) on a 12-h light-dark cycle with free access to food and water. According to experiments, mice were fed ad libitum with a standard (22.4 % fat, 60.9 % carbohydrate and 16.7% protein; 2.9 Kcal/g; R04T25, Safe, France) or high-fat diet (42.5 % fat, 42.5 % carbohydrate and 15 % protein; 4.4 Kcal/g; Customized, Safe, France). For fasting group, food was removed just before dark onset (19h00). For tissue collection, mice were killed by cervical dislocation. Brains were quickly removed and immediately immersed in ice-cold PBS containing 5 mM HEPES.

**Surgery.** Under anesthesia (0.4% isoflurane – 100% O₂), mice underwent stereotaxic surgery to implant a chronic stainless steel cannula (Charles Rivers). The 3rd-cerebral ventricle was targeted using the following coordinates from Bregma: anterior-posterior, -0.825 mm; dorsal-ventral, -5 mm; and medial-lateral, 0 mm. The cannula was fixed to the skull using dental cement. Mice were finally housed individually and were allowed 1 week for recovery before experiment.

**Drug administration.** Injections into the 3rd-cerebral ventricle were performed in awake mice. The injections (1 µl) consisted of either 0.4 µU insulin (Recombinant Human Insulin, Actrapid 100 U/ml, Novo Nordisk), 0.1 mM trolox (Calbiochem), 0.1 mM diphenyleneiodonium (DPI, Sigma-Aldrich) or vehicles. Insulin, trolox and DPI were prepared as stock solutions in “diluting medium for soluble insulin injection” (Novo Nordisk), 1% ethanol, and 1% DMSO, respectively. For injections, all drugs and their vehicles were diluted in 0.9 % NaCl. Injections were performed over 1 min. Following the infusion, the guide cannula was kept in place for an additional 30 s to allow the drugs to diffuse away from the cannula tip.

**Food intake measurement.** Food was removed 1 h before intracerebroventricular (icv) injection. Insulin was injected 4 h before the dark onset (15h00). Icv injections of trolox or DPI were performed 30 min prior 0.4 µU insulin injection. Just prior the beginning of dark period (19h00), food (10 g) was presented to mice. 12 and 24 h later, food intake and body weight were measured.

**Blood glucose measurement.** Blood samples were collected from the tail 2 h after 4 µU insulin icv injection and used for measurements of blood glucose. It was directly measured on a glucose analyzer (ACCU-CHEK Active).

**Reactive oxygen species (ROS) detection.** The dye 2’,7’-dichlorofluorescein diacetate (H₂DCFDA, Molecular Probes) is used to monitor intracellular change in H₂O₂ level. After icv injections, hypothalami were dissected and immediately frozen in liquid nitrogen. After rapid thawing, tissues were homogenized (20 strokes) with a dounce homogenizer and a B-type pestle in 250 µl of a ROS-buffer (150 mM KCl, 20 mM tris, 0.5 mM EDTA, 1 mM MgCl₂, 5 mM glucose, 0.5 mM octanoic acid, pH 7.4). Homogenates were exposed to 16 µM H₂DCFDA and were incubated at 37°C for 30 min under agitation. Reaction was stopped with 125 µl of 70 % ethanol plus 125 µl of 0.1 N HCl. Homogenates were then centrifuged at 3,000 g for 15 min at 4°C. Supernatants were collected, neutralized with 175 µl of 1 M NaHCO₃ and centrifuged at 6,000 g for 15 min at 4°C. As already described (9-10), ROS level was evaluated by measuring fluorescence intensity with a microplate reader (Victor; Wallac). Intensity of fluorescence was expressed as arbitrary units.
Reduced glutathione assay. Dissected hypothalami were immediately frozen in liquid nitrogen. Tissues were homogenized (20 strokes) with a dounce homogenizer and a B-type pestle in a lysis saline solution (150 mM KCl, 3 mM EDTA, pH 7.4). Homogenates (50 µl) were mixed with 450 µl of 5 % metaphosphoric acid. Samples were then centrifuged at 1500 g for 10 min at 4°C. Supernatants were collected and used to detect reduced glutathione (GSH). Glutathione assay was performed by reverse-phase high-performance liquid chromatography (HPLC) as previously described (21). The results were expressed as fmol/µg tissular proteins.

Protein assay. Protein concentration of samples was determined using the DC Protein Assay Kit (Bio-Rad SA) according to the manufacturer’s instructions.

Statistical analysis. Data are given as means ± SEM. According to experiments, data were compared by unpaired Student’s t test, Mann-Whitney U test or one-way analysis of variance (ANOVA). Differences among groups were considered significant when p was < 0.05.

RESULTS

Third ventricle insulin injection decreases food intake. In order to study the role of hypothalamic insulin signaling in food intake regulation, we first aimed to select the best paradigm of acute insulin injection (Figure 1A). We chose a 3rd ventricle injection because acute insulin administration into the lateral ventricle was without effect on food intake in mice (22). In comparison to vehicle-injected animals, insulin significantly reduced food intake 12 and 24 hours after injection (Figure 1B). This represented a 40 % decrease in daily food intake. Concurrently to this food intake inhibition, insulin also significantly reduced body weight at 12 and 24 hours (Figure 1C). To ensure that icv injected insulin did not indirectly affect food intake through diffusion out of the brain, we injected a higher dose of insulin and measured glycemia. No change in plasma glucose was observed in this condition, showing that the insulin effect on food intake only depends on its effect on the brain (Figure 1D).

Insulin induces a transient hypothalamic ROS increase which is required for food intake inhibition. In order to determine whether insulin could induce ROS increase in the hypothalamus, insulin was injected into the 3rd ventricle at the dose that inhibits food intake. Mice were killed 5, 15 and 30 minutes later and ROS level was evaluated by using the fluorescent redox-sensitive dye H2DCFDA. Figure 2A shows that insulin induced a significant increase (+36 %) in ROS level 15 minutes post-injection. This insulin-induced ROS increase was transient, as no modification of fluorescence intensity was observed either at 5 or 30 minutes post-injection.

We then designed experiments to examine the physiological relevance of this insulin-induced hypothalamic ROS elevation. For this purpose, we assessed food intake in response to acute icv insulin injection in combination with an icv pretreatment of trolox, a ROS scavenger. We first verified that trolox alone did not induce significant change in basal ROS level and food intake, and then we investigated the ability of trolox to inhibit the insulin-induced ROS production. As illustrated in figure 2B, trolox delivery 30 minutes prior insulin completely prevented insulin-stimulated ROS increase. We then measured the effect of trolox pretreatment on food intake response. As for the ROS level, icv trolox injection completely suppressed insulin-induced food intake inhibition without significantly modifying basal food intake in trolox-injected mice (Figure 2C). Therefore, these results indicate that the transient insulin-
induced ROS elevation in the hypothalamus is required for food intake inhibition.

**NADPH oxidase is required for the insulin-induced ROS elevation and food intake inhibition.** In peripheral tissues, ROS produced by membrane-bound NADPH oxidase are involved in the insulin signaling cascade. We questioned whether, as in periphery, the NADPH oxidase was involved in the hypothalamic insulin ROS signaling to regulate food intake. For this purpose, we assessed food intake in response to acute icv insulin injection in combination with a prior icv administration of diphenyleneiodonium (DPI), a NADPH oxidase inhibitor. To test the ability of DPI to inhibit the ROS level increase, we first measured the hypothalamic ROS level 15 minutes after insulin injection with DPI pretreatment. DPI delivery was effective in fully preventing insulin-stimulated ROS elevation (Figure 3A). In this condition, DPI completely restored basal food intake in insulin-injected mice (Figure 3B). DPI treatment alone did not significantly modify basal ROS level as well as food intake. Therefore, these results indicate that the NADPH oxidase-dependent ROS increase within the hypothalamus is required for the central effect of insulin on the food intake inhibition.

**Impairment of insulin-induced food intake inhibition is associated with a lack of hypothalamic ROS level increase.** We then tested whether a modification of insulin effect on food intake was systematically associated with a change of ROS level within the hypothalamus. For this purpose, we chose two physiological situations that produce central insulin unresponsiveness: 18 hours fasting (energy deficiency, 20) and 3 days high-fat diet (energy excess, 7). Figure 4A shows that these two conditions effectively suppressed the insulin-induced food intake inhibition. According to our hypothesis, insulin did not promote any more elevation of ROS level within the hypothalamus 15 min after icv injection (Figure 4B).

As cellular ROS level results from the balance between their production and removal by various antioxidants, we quantified the hypothalamic glutathione that is considered as a major cellular ROS scavenging system (23). Figure 4C shows that hypothalamic reduced glutathione (GSH) level was increased by 18 hours fasting (+20%) and 3 days high fat diet (+36%, p < 0,05).

Therefore impairment of insulin-induced inhibition of food intake is associated with a lack of ROS increase in the hypothalamus likely due to higher GSH level. These results strengthen the crucial role of hypothalamic ROS signaling in the anorexigenic effect of insulin.

**DISCUSSION**

In the present study, we describe the involvement of a NADPH oxidase-dependent ROS signaling pathway in the anorexigenic insulin effect. We demonstrate for the first time the physiological relevance of this concept in conscious mice.

Insulin was administrated into the third ventricle at a dose (2.4 nM) that is consistent with the brain insulin receptor affinity (24-25) and that produces maximal food intake inhibition in mice (2 and unpublished results). At this physiological concentration, insulin induces a transient ROS level elevation within the hypothalamus (Figure 2). The present data demonstrates for the first time *in vivo* that insulin can effectively induce a change in ROS level in the central nervous system. Despite a relatively low magnitude (+36 %), this ROS level increase is similar to that observed in the hypothalamus after glucose, lipid, or ghrelin stimulation (9-11). These subtle ROS changes play a key role in the control of autonomous nervous system and food intake. We show that this insulin-induced ROS increase is required for food intake inhibition,
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thus demonstrating its physiological relevance. First, the suppression of insulin-stimulated ROS production by using pharmacological tools prevented insulin inhibition of food intake. Second, in two nutritional conditions of central insulin unresponsiveness (i.e. 18 hours fasting or 3 days high-fat diet), impairment of the insulin effect on food intake was associated with a lack of insulin-stimulated ROS elevation within the hypothalamus. These data strongly support the notion that cellular ROS constitute a pivotal second messenger in hypothalamic signaling pathways involved in energy homeostasis regulation, especially by controlling nutrients sensing (9-10) and hormonal signaling (11).

The involvement of an oxidant signal in the action of insulin has been suggested for decades, mainly with the observation that oxidants, including hydrogen peroxide, mimic insulin effects on glucose transport and lipogenesis in adipose cells (26). A few years later, insulin itself was shown to elicit generation of hydrogen peroxide in adipocytes (27). More recently, several elegant studies carried out by Mahadev et al. clearly demonstrate that a physiological transient insulin-induced ROS elevation mediates increase of glucose uptake though Glut4 translocation to the plasma membrane in adipocytes (13-16). Finally, creating mildly and transiently oxidative intracellular conditions has been reported to enhance the insulin receptor activation, suggesting that optimal insulin responsiveness involved a process of “redox priming” of the β subunit (17, 28). Our results reinforce these previous data. Altogether, they strongly support the idea that a ROS increase induced by insulin i) forms an integral part of insulin signaling pathway in the central nervous system as in periphery and ii) is relevant in the whole body energy homeostasis.

The observed insulin-induced ROS increase peaks and dissipates in few minutes. Nevertheless, and in agreement with the study by Brown et al., feeding behavior is significantly inhibited during several hours after acute icv insulin injection (2, figure 1). In adipocytes, the early insulin-stimulated production of ROS is required for insulin to elicit its late cellular responses, especially by activating the IRS/PI3K signaling pathway (13-16, 18, 29). Within the hypothalamus, both insulin-induced ROS and IRS/PI3K signaling pathways are essential for long-term inhibitory insulin effect on food intake (Figure 2, 30). Reciprocally diet-induced impairment of insulin effect on food intake is associated with an alteration of these two insulin-activated signaling pathways (Figure 4A-B, 7, 31). The time course of insulin-induced ROS level increase is similar to that observed for the insulin-induced PI3K activation in the hypothalamus, with activation in few minutes after stimulation, persisting for 10-15 minutes and gradually declining over 30-60 minutes (32). Altogether, these data strongly suggest that the early insulin-induced ROS increase that we measured within the hypothalamus is a pivotal component of the insulin-induced IRS/PI3K signaling pathway activation, leading to a long-term decrease in food intake, likely through the modulation of genes expression such as neuropeptide Y (33). Actually, hypothalamic neuropeptide Y and pro-opio melanocortin C are well-known to play a key role in food intake regulation and to be down or up-regulated by hormones such as leptin, ghrelin or insulin (2, 11, 33-34). As the insulin-induced PI3K activation occurs in these neuronal subpopulations (32), one can speculate that the insulin-induced ROS increase occurs at least in one of these, as it has been recently demonstrated for ghrelin (11).

The fact that insulin induces a rapid and transient increase in ROS level within the hypothalamus leads us to investigate the involvement of NADPH oxidase. Indeed, this
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enzyme can be rapidly activated by insulin, leading to a rapid and transient ROS increase in peripheral cells (14-15). In our experimental conditions and similarly to Mahadev et al. (15), the pretreatment with a NADPH oxidase inhibitor (DPI) completely abolishes i) the rapid and transient insulin-induced ROS increase within the hypothalamus and ii) the inhibitory insulin effect on food intake (Figure 3). We can thus conclude that NADPH oxidase plays a critical role in insulin signaling pathway in the brain as in periphery. There are now compelling evidences reporting the presence of NADPH oxidase homologues in rodents or human brain tissue (35-38). Within the hypothalamus, immunoreactivity for NADPH oxidase subunits, such as p47phox and gp91phox, was found in arcuate, ventromedian and paraventricular nuclei that are well known to be involved in energy homeostasis regulation (36). Moreover, increasing data demonstrate the importance of redox signaling derived from NADPH oxidase in normal central nervous system processes, such as the long-term potentiation and hippocampal-dependent memory or the regulation of the cardiovascular system (38-42). Further in vivo studies would permit to confirm that an acute insulin-induced NADPH oxidase activation mediates other hypothalamic insulin effects involved in energy homeostasis.

After fasting or short-term high-fat feeding, the brain became less responsive to insulin which results in a loss of its effects on food intake (Figure 4A, 7, 20). In this context, the insulin-induced hypothalamic ROS elevation was suppressed (Figure 4B). To explain this result, we explored the involvement of antioxidant defence systems. Indeed, the raised in free fatty acids availability during fasting (due to the lipolysis) and short-term high-fat feeding (due to the diet) likely induced an increase in hypothalamic lipid oxidation (9, 11, 43), a mechanism known to produce ROS (44). In physiological range, excess of ROS is scavenged at cellular level by stimulating the synthesis of antioxidant defence systems (44), as already demonstrated within the hypothalamus of food deprived animals (9, 45). We reported that hypothalamic GSH level is effectively higher after high-fat feeding and to a lesser measure after fasting (Figure 4C), with a concomitant hypothalamic normal ROS level. These data strongly suggest that i) diets have been induced a hypothalamic excessive ROS production which has been well regulated by cellular scavenging system, and ii) this increased antioxidant defence system could consequently over-quench ROS produced under insulin stimulation. Such cellular mechanism has been already proposed to explain the impaired peripheral insulin sensitivity observed during physiological conditions such as pregnancy (46). In animal models of long-term high-fat diet-induced obesity (47-48) associated with hypothalamic insulin resistance (47), oxidative stress appears in the brain (48). This cellular stress has been proposed to be critical mechanism underlying development of insulin resistance in obesity (49, 50). We thus hypothesized that i) the early diet-induced central insulin resistance is a consequence of an adaptive mechanism to avoid cytotoxic ROS production although ii) in the long-term, the resistance might be caused by overwhelming of antioxidant defence systems, and thus oxidative stress (44).

In conclusion, we report that insulin injected into the third ventricle promotes a subtle and transient increase in ROS level within the hypothalamus. This mechanism is required for insulin food intake inhibition and involves the NADPH oxidase (Figure 5). We thus suggest that i) a NADPH oxidase-dependent ROS-sensitive signaling pathway is implied in hypothalamic insulin action, and ii) diet-induced impairment of hypothalamic
ROS homeostasis may contribute to the cerebral insulin resistance.

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FIGURES LEGENDS

FIG. 1. Acute insulin injection into the 3rd ventricle reduces food intake at 12 and 24 h with no effect on plasma glucose. A. Schematic representation of experimental procedures of food intake measurement. B. Mice were injected into the 3rd ventricle with insulin (0.4μU) or vehicle. Food intake was assessed by weighing residual chow 12 and 24 h after food presentation (n= 9-10 mice per group). C. Body weight was also measured at these time points (n= 9-10 per group). D. Mice were injected into the 3rd ventricle with a higher dose of insulin (4μU) or vehicle. Plasma glucose was measured 2 h after injection (n= 4 per group). Data are means ± S.E.M. *P<0.05, **P < 0.01 vs vehicle-injected group.

FIG. 2. Insulin induces a transient hypothalamic ROS increase which is required for food intake inhibition. A. Insulin effect on ROS production. After icv injection of insulin (0.4μU) or vehicle, ROS levels were assessed by oxidation of H2DCFDA probe in the hypothalamus and at different times (n= 5 mice per group). DCF fluorescence is expressed in arbitrary units per µg of proteins. B,C. Effect of trolox on insulin-induced ROS production and food intake inhibition. Trolox (0.1mM) was icv injected 30 min before insulin (0.4μU). ROS levels were assessed in the hypothalamus 15 min after injections (n= 5 per group) (B). Food intake was assessed 12 h after food presentation (n= 5-10 per group) (C). Data are given as means ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 vs vehicles- or insulin-injected group.

FIG. 3. NADPH-oxidase is required for the insulin-induced hypothalamic ROS increase and food intake inhibition. Effect of DPI on insulin-induced ROS level change and food intake inhibition. DPI (0.1mM) was icv injected 30 min before insulin (0.4μU). ROS levels were assessed in the hypothalamus 15 min after injections (n= 10 animals per group) (A). Food intake was assessed 12 h after food presentation (n= 5-10 per group) (B). Data are given as means ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 vs vehicles- or insulin-injected group.

FIG. 4. Impairment of insulin-induced food intake inhibition is associated with a lack of ROS level elevation within the hypothalamus. Effect of 18 hours fasting or 3 days high-fat diet on insulin-induced food intake regulation and ROS production, and on glutathione levels. A-B. Mice were injected with insulin (0.4μU) or vehicle. Food intake (A) (n= 8-12 animals per group) and ROS level (B) (n= 5-10 per group) were assessed 12 h and 15 min after insulin injection respectively. *P < 0.05, **P < 0.01 vs vehicle-injected mice fed with standard diet. C. Hypothalamic reduced glutathione (GSH) level was measured in standard, fasted and high-fat diet fed mice (n= 5-10 per group). *P < 0.05 vs mice fed with standard diet.

FIG. 5. Proposed mechanism of the involvement of a hypothalamic NADPH oxidase-dependent ROS signaling pathway in the anorexigenic effect of insulin. Insulin binds to its receptor leading to NADPH oxidase activation and generation of ROS. This ultimately leads to inhibition of food intake, likely though modulation of genes expression. IR indicates insulin receptor; DPI, diphenylenedioiodonium; ROS, reactive oxygen species.
ROS-mediated anorexigenic insulin effect

Figure 1.
Figure 2.

Figure 3.
Figure 4.

A

B

C

ROS-mediated anorexigenic insulin effect

Figure 4.
Figure 5.