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Review Article

The effect of insulin-induced hypoglycemia on inflammatory markers: A systematic review

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ABSTRACT

Introduction: The effects of acute hypoglycemia on markers of inflammation have been investigated, but the results have been heterogeneous.
Objective: We aimed to perform a systematic review about the acute effects of insulin-induced hypoglycemia on inflammatory markers in patients with diabetes as well as non-diabetic subjects.
Methods: A systematic search of the literature using the electronic databases MEDLINE and SCOPUS was conducted through September 2017. Search terms included: "hypoglycemia"," insulin", "cytokines", and "inflammation". We included original studies assessing peripheral inflammatory markers during insulin-induced hypoglycemia in humans.
Results: Two hundred twenty-two citations were initially retrieved. Eleven studies were included in our systematic review. Acute hypoglycemia increases total leukocyte number and several pro-inflammatory markers. Elevation in pro-inflammatory markers in response to insulin-induced acute hypoglycemia appears to be of similar magnitude in non-diabetic subjects and in type-1 diabetic patients with intact awareness of hypoglycemia. Adrenaline rises in response to acute hypoglycemia correlates with the increase of pro-inflammatory markers.
Conclusion: Acute hypoglycemia induces a pro-inflammatory state in both type-1 diabetic and non-diabetic subjects with no apparent significant difference between these two populations. Activation of the sympathetic nervous system is a likely mediator of these effects.

1. Introduction

Hypoglycemia is an important stress event to the body. The counter-regulatory responses aiming to restore normoglycemia involve increases in blood levels of glucagon, adrenaline, noradrenaline, cortisol, and growth hormone (Mitrakou et al., 1991). In addition to these classical counter-regulatory responses, hypoglycemia appears to activate an inflammatory response in adults with or without diabetes. This response is characterized by the elevation of inflammatory molecules such as C reactive protein (CRP), interleukin (IL)-6 and tumor necrosis factor (TNF) in the circulation (Joy et al., 2016; Razavi Nematollahi et al., 2009; Wright et al., 2010). In addition, hypoglycemia also increases the number of circulating leukocytes (Frier et al., 1983; Ratter et al., 2017).

Insulin-induced hypoglycemia can be achieved in individuals with or without diabetes using either the hyperinsulinemic hypoglycemic clamp or the insulin tolerance test (ITT). The hyperinsulinemic hypoglycemic clamp is a variant of the glucose clamp procedure used to evaluate the physiological counter-regulatory hormone responses under standardized conditions of experimental hypoglycemia (Boyle, 1994). The hyperinsulinemic hypoglycemic clamp has been widely used in the research setting to investigate the pathophysiology of iatrogenic hypoglycemia, to study the effects of different anti-diabetic drugs in the endogenous counter-regulatory response in both type-1 and type-2 diabetic populations (Farngren et al., 2014; Pieber et al., 2015), and also to investigate the impact of hypoglycemia unawareness on brain responses in type-1 diabetic patients (Maran et al., 2017). The ITT is a dynamic test that consists of a single intravenous bolus of regular insulin leading to a rapid fall in blood glucose concentration. In the clinical practice, the ITT is regarded as the gold-standard procedure for

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the evaluation of the reactivity of hypothalamic-pituitaryadrenal axis (HPA) and the growth hormone secretory capacity (Erturk et al., 1998; Hazem et al., 2011), but various additional stress-related parameters can also be assessed under acute hypoglycemic stimulus, such as vasopressin, prolactin, glucagon and catecholamines.

Heterogeneous results regarding the effects of acute hypoglycemia on markers of inflammation have been reported in both diabetic and non-diabetic subjects, and different factors have been implicated. For instance, diabetic patients who undergo supervised insulin-induced hypoglycemia should avoid hypoglycemic episodes at least 24 h antedating the procedure and normoglycemia needs to be obtained before the induction of hypoglycemia (Ceriello et al., 2013a; Wright et al., 2010). Other factors such as clinical comorbidities, duration of diabetes and the degree of metabolic control may also interfere with the counter-regulatory response (Bolli et al., 1984). The magnitude of the inflammatory response may be related to the onset and duration of the hypoglycemic stimulus (Dandona et al., 2010). In addition, it is not clear yet whether the inflammatory response to acute hypoglycemia is different in the diabetic population in comparison to non-diabetic subjects. Available data indicate that the extent of the inflammatory reaction to acute hypoglycemia in diabetic patients with impaired awareness of hypoglycemia (IAH) differs from those observed in diabetic patients with normal awareness of hypoglycemia (NAH) and in non-diabetic individuals (Ratter et al., 2017). Diabetic patients with IAH represent indeed a specific population who are at greater risk of severe hypoglycemia (Frier, 2008) as they do not exhibit the classical warning symptoms of hypoglycemia (sweating, tachycardia, anxiety) due to an attenuated adrenaline response (Cryer, 2013).

Besides the heterogeneity of the available results on the inflammatory response to insulin-induced acute hypoglycemia, its biological meaning (i.e. counter-regulatory response?) and its potential modulators (e.g. adrenaline, others?) have not been fully elucidated. Moreover, it has been speculated that an attenuated pro-inflammatory response to acute hypoglycemia could be an adaptive reaction to repeated episodes of hypoglycemia (Ratter et al., 2017). In this context, we carried out a systematic review aiming to analyze the acute effects of insulin-induced hypoglycemia on inflammatory markers in diabetic patients as well as non-diabetic subjects.

2. Methods

2.1. Search strategy and study selection criteria

A systematic search of the literature using the electronic databases MEDLINE, SCOPUS and SCIELO was conducted through September 2017. The search terms included: “hypoglycemia”, “insulin”, “cytokines”, and “inflammation”. There was no restriction regarding the date of publication. Studies written in English, Portuguese or Spanish were selected for review.

Two reviewers independently evaluated the titles and abstracts, and then the full text for inclusion eligibility (J.B.D. and I.G.B.). Disagreements were evaluated by arbitration with a third reviewer (A.L.T.). Only original studies assessing peripheral inflammatory markers during insulin-induced hypoglycemia in humans were eligible for inclusion.

2.2. Data extraction process and literature quality assessment

We developed a data extraction table based on the Cochrane template (Chandler et al., 2015). One investigator (J.B.D) extracted the data and a second reviewer (I.G.B.) verified the extracted data. In addition, two reviewers (J.B.D. and I.G.B.) independently cross-checked the risk of bias using the Newcastle-Ottawa Scale for observational studies (Wells et al., 2011). The Newcastle-Ottawa form assigns a maximum of four stars for selection, two stars for comparability and three stars for exposure or outcome. In the current study, we considered a study awarded seven or more stars as a high-quality study (Yuhara et al., 2011). Any disagreement between authors was resolved by consensus, if necessary a third author (A.L.T.) was consulted.

The data extracted included publication data (the first author’s last name, year of publication and country of the population studied), type of study, characteristics of the study population (sample size, mean age and sex and specific characteristics of the diabetic population), characteristics of the study protocol (hypoglycemia induction protocol, depth and duration of hypoglycemia, measured inflammatory markers, laboratory methodology and timed blood collections) and main outcomes.

3. Results

3.1. Description of the studies

A total of 222 studies were identified through database search (PUBMED: 113, SCOPUS: 109). Duplicate articles were excluded (N: 25) and 187 studies were further excluded after title and abstract screening. Ten additional articles were identified through reference lists. Of the 20 articles selected for full text review, nine were excluded (3 studies were not conducted in humans, 3 articles evaluated the connection between hypoglycemia and inflammation through distinct protocols other than acute insulin-induced hypoglycemia and three studies evaluated exclusively non-inflammatory endothelial function markers). A total of 11 studies were selected for this review (Fig. 1).

3.2. Characteristics of included studies

The 11 selected studies comprised ten case-control studies (Ceriello et al., 2013a,b; de Galan et al., 2003; Dotson et al., 2008; Frier et al., 1983; Galloway et al., 2000; Gogitidze Joy et al., 2010; Joy et al., 2016; Ratter et al., 2017; Wright et al., 2010) and one case series (Razavi Nematollahi et al., 2009). The quality of the case-control studies evaluated by Newcastle-Ottawa Score was assessed (Table 1). The mean value for the ten studies assessed was five, indicating a low to moderate overall quality.

Eight studies employed the hyperinsulinemic hypoglycemic clamp (Ceriello et al., 2013a,b; de Galan et al., 2003; Dotson et al., 2008; Gogitidze Joy et al., 2010; Joy et al., 2016; Ratter et al., 2017; Wright et al., 2010) while three studies used the standard ITT (Frier et al., 1983; Galloway et al., 2000; Razavi Nematollahi et al., 2009). Hypoglycemic targets varied between studies, ranging from < 40 mg/dl (2.2 mmol/L) to < 52 mg/dl (2.9 mmol/L). Studies that used the hyperinsulinemic hypoglycemic clamp usually compared the obtained results with the control group (hyperinsulinemic normoglycemic clamp). One of these studies also compared the inflammatory response elicited by the hyperinsulinemic hypoglycemic clamp with the results observed with hyperglycemic clamps, either normoinsulinemic or hyperinsulinemic (Joy et al., 2016).

The samples included in these studies were very heterogeneous. Four studies evaluated only non-diabetic individuals (Dotson et al., 2008; Frier et al., 1983; Joy et al., 2016; Razavi Nematollahi et al., 2009) (Table 2) while seven studies included type-1 diabetes mellitus (T1DM) patients (Table 3). Five of these latter studies were conducted in both T1DM patients and non-diabetic subjects (de Galan et al., 2003; Gogitidze Joy et al., 2010; Ratter et al., 2017; Wright et al., 2010) and the remaining two studies included only T1DM patients (Ceriello et al., 2013a,b). None of the studies included type-2 diabetes mellitus (T2DM) patients.

The timing of blood collections for biomarker assessment also varied significantly among studies. The majority (9/11) of the studies included blood collection within the first 240 min but a few (Galloway et al., 2000; Wright et al., 2010) extended the collection period up to 24 h post-hypoglycemia.

Results of variations of inflammatory markers evaluated under acute hypoglycemia are summarized below.

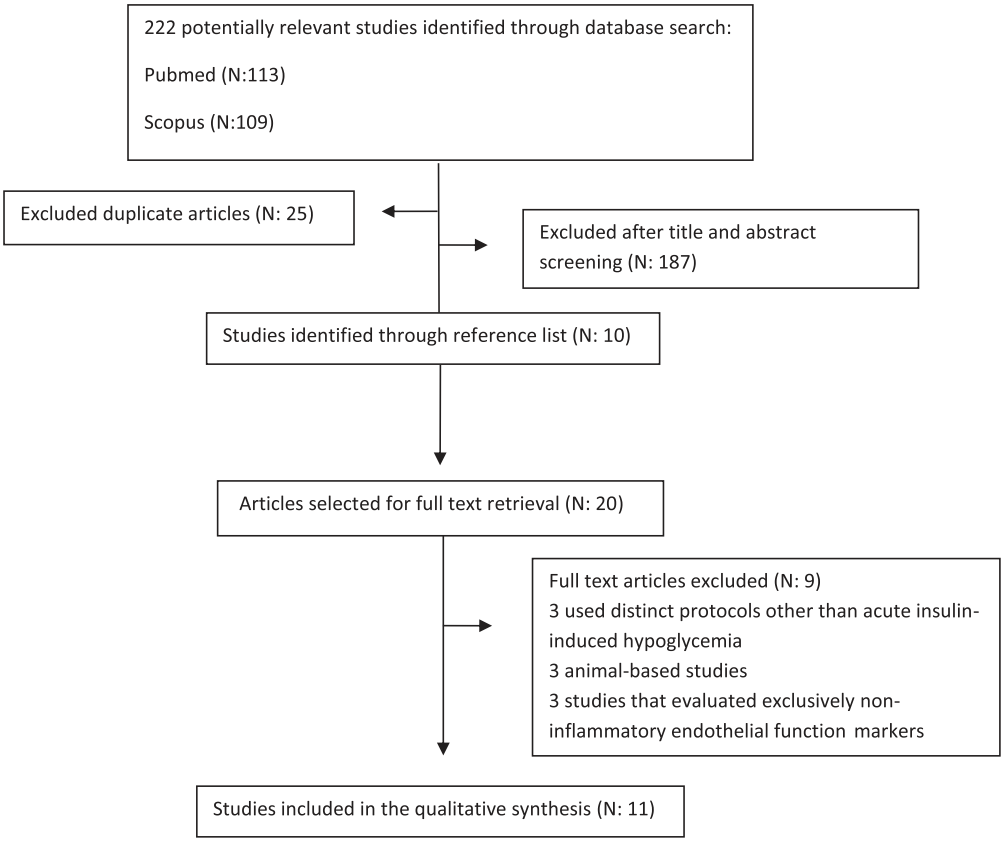

Fig. 1. The process of study selection

3.3. Peripheral leukocyte counts

One study showed that hypoglycemia was able to elicit an increase in total leukocyte count in non-diabetic subjects, characterized by initial lymphocytosis followed by neutrophilia (Frier et al., 1983). Two other studies corroborated this finding of increased leukocyte number after acute hypoglycemia in non-diabetic subjects (Ratter et al., 2017; Razavi Nematollahi et al., 2009).

The peak value of leukocyte numbers was associated with elevated cortisol and noradrenaline levels (Ratter et al., 2017; Razavi Nematollahi et al., 2009). Ratter and collaborators also showed that acute hypoglycemia increased total leukocyte numbers in T1DM patients with NAH, but not in those with IAH (Ratter et al., 2017). Moreover, this increase in total leukocytes number was mainly due to an increase in lymphocytes instead of monocytes.

Cluster of differentiation 40 (CD40) expression by circulating monocytes was found to increase after hypoglycemia as compared to normoglycemia in both T1DM patients and in non-diabetic subjects (Wright et al., 2010).

Table 1
Quality of the case control studies evaluated by Newcastle-Ottawa Score[a].

First author (reference)	Year	Selection				Comparability[b]	Exposure			Overall Quality Score[c]
		Is the case definition adequate?	Represen-tativeness of the cases	Selection of controls	Definition of controls		Ascertainment of exposure	Same method of ascertain-ment for cases and controls	Non-Response rate	
Ratter[5]	2017	⋆	0	0	⋆	⋆	⋆	⋆	0	5
Joy[3]	2016	⋆	0	0	⋆	0	⋆	⋆	0	4
Ceriello[9]	2013	⋆	0	⋆	⋆	⋆⋆	⋆	⋆	0	7
Ceriello[21]	2013	⋆	0	⋆	⋆	⋆⋆	⋆	⋆	0	7
Wright[4]	2010	⋆	0	0	⋆	⋆	⋆	⋆	0	5
Gogitidze Joy[20]	2010	⋆	0	0	⋆	⋆	⋆	⋆	0	5
Dotson[19]	2008	⋆	0	0	⋆	⋆	⋆	⋆	0	5
de Galan[18]	2003	⋆	0	0	⋆	0	⋆	⋆	0	4
Galloway[17]	2000	⋆	0	0	⋆	0	⋆	⋆	0	4
Frier[6]	1983	⋆	0	0	⋆	0	⋆	⋆	0	4

[a] A study can be awarded a maximum of one star for each numbered item except for item Comparability.
[b] A maximum of two stars can be awarded for Comparability.
[c] Maximum = 9.

Table 2
Studies evaluating pro-inflammatory markers including only non-diabetic subjects.

Reference (Country)	Type of study	Study Population	Study Characteristics					Main outcomes
			Hypoglycemia induction protocol	Depth and duration of hypoglycemia	Measured inflammatory biomarkers	Laboratory methodology	Timed blood collections	
Joy et al., 2016 (USA)	Case-control	45 non-diabetic subjects (21M, 38 ± 3 y)	Normoinsulinemic hyperglycemia X Hyperinsulinemic hyperglycemia X Hyperinsulinemic normoglycemia X Hyperinsulinemic hypoglycemia	Mean glucose nadir of 2.9 ± 0.01 mmol/L for 120 min	Plasma IL6, TNF	LINCO research kits (IL6, TNF)	Basal and at the last 30 min of the clamp	Responses of IL-6 and TNF were increased during hypoglycemia as compared to normolycemia or hyperinsulinemic hyperglycemia ($p < 0.05$)
Razavi Nematollahi et al., 2009 (USA/Iran)	Case series	13 non-diabetic subjects (13M, 30 ± 4.8 y)	Intravenous bolus of regular insulin	Mean glucose nadir 2.12 ± 0.24 mmol/L (at 30 min after insulin injection)	Plasma TNF, IL-6, IL-8, IL1-β, CRP WBC counts	Immulite CLIA (TNF, IL-6, IL-8, IL1-β, CRP)	Basal +30, +45, +60, +120, +240 after insulin injection	Insulin-induced hypoglycemia was associated with increased levels of TNF, IL-6, IL-8 ($p < 0.05$) and increased number of WBC counts ($p < 0.001$)
Dotson et al., 2008 (USA)	Case-control	17 non-diabetic subjects (8M, 28.9 ± 2 y)	Hyperinsulinemic normoglycemia X Hyperinsulinemic hypoglycemia	Mean glucose nadir of 2.79 ± 0.2 mmol/L at 30 min and maintained for approximately 60 min	Serum IL6	ELISA	−15, 0+, 45, +75, +105, +135 min after insulin infusion	IL-6 levels increased during hypoglycemia as compared to normoglycemia ($p < 0.001$)
Frier et al., 1983 (UK)	Case-control	6 non-diabetic control subjects (6M) X 5 non-diabetic tetraplegic subjects (5M) X 6 splenectomized subjects (5M)	Intravenous bolus of regular insulin with or without B-blockade	Mean glucose nadir: 0.9 ± 0.05 mmol/L (at approximately 30 min after insulin injection)	Total and differential WBC count	Measurement of hematological parameters (Model S Coulter-counter)	Basal, +15, +30, +60,+ 90,+120, +150,+180, +210 min after insulin injection	Acute hypoglycemia elicited immediate lymphocytosis followed by neutrophilia ($p < 0.05$). The early lymphocytosis was absent in sympathectomized subjects ($p < 0.01$) and reduced under beta-blockade ($p < 0.02$)

M: males; y: years-old; IL1β: interleucin 1β, IL6: interleucin 6, IL8: interleucin 8; CRP: C-reactive protein PAM; TNF: tumor necrosis factor, WBC: white blood cells; PBMCs: peripheral blood mononuclear cells; CLIA: chemiluminescence immuno assay ; ELISA: enzyme-linked immunosorbent assay UK: United Kingdom; USA: United States of America.

3.4. C Reactive protein (CRP)

Serum CRP levels were measured after insulin intravenous bolus administration (Galloway et al., 2000) and after hyperinsulinemic clamp (Wright et al., 2010). One study showed increased CRP serum levels after acute hypoglycemia in non-diabetic subjects and T1DM patients (Galloway et al., 2000). The other study showed higher CRP serum levels in a group of non-diabetic subjects during hypoglycemia when compared to normoglycemia (Wright et al., 2010).

3.5. Interleukin 6 (IL-6)

Interleukin 6 was the most frequently evaluated cytokine. Eight studies showed increased IL-6 plasma levels in response to acute hypoglycemia, either after hyperinsulinemic clamp (Ceriello et al., 2013a,b; de Galan et al., 2003; Dotson et al., 2008; Gogitidze Joy et al., 2010; Joy et al., 2016; Wright et al., 2010) or insulin intravenous bolus administration (Razavi Nematollahi et al., 2009).

A recent study showed increased IL-6 plasma levels in non-diabetic subjects after hyperinsulinemic hypoglycemia when compared with hyperinsulinemic normoglycemia or hyperinsulinemic hyperglycemia (Joy et al., 2016). These findings are supported by two independent studies that reported increased IL-6 plasma levels after acute hypoglycemia in non-diabetic subjects when compared with normoglycemia (Dotson et al., 2008; Razavi Nematollahi et al., 2009). Notwithstanding, one study was unable to show any effect of hypoglycemia on IL-6 plasma levels in non-diabetic subjects (de Galan et al., 2003).

One study including both T1DM patients and non-diabetic subjects reported increased IL-6 plasma levels in response to acute hypoglycemia as compared with normoglycemia in both populations (Gogitidze Joy et al., 2010). In a different study, significant increases of IL-6 plasma levels were also reported after hypoglycemia as compared with baseline in both T1DM patients and non-diabetic subjects. However, a similar increase of IL6 plasma levels was documented after normoglycemic hyperinsulinemia in this study (Wright et al., 2010). Two studies evaluated only T1DM patients and were able to report increased IL-6 plasma levels after acute hypoglycemia when compared with baseline levels (Ceriello et al., 2013a,b), with similar elevations of IL-6 plasma levels after either hypoglycemia or hyperglycemia (Ceriello et al., 2013a).

3.6. Tumor necrosis factor (TNF)

TNF, formerly known as TNFα, was measured after hyperinsulinemic clamp (de Galan et al., 2003; Gogitidze Joy et al., 2010; Joy et al., 2016) and after insulin intravenous bolus administration (Razavi Nematollahi et al., 2009). Two studies evaluating non-diabetic subjects (Joy et al., 2016; Razavi Nematollahi et al., 2009) showed increased TNF plasma levels in response to acute hypoglycemia. One study including T1DM patients (Gogitidze Joy et al., 2010) also reported increased TNF plasma levels after hypoglycemia when compared with normoglycemia. One study including non-diabetic subjects did not find any significant change in circulating TNF plasma levels after acute hypoglycemia (de Galan et al., 2003).

3.7. Other cytokines

Plasma levels of IL-1β response to acute hypoglycemia were only evaluated in non-diabetic subjects. Two studies reported no significant changes in IL-1β plasma levels after acute hypoglycemia (de Galan et al., 2003; Razavi Nematollahi et al., 2009). However the measurement of circulating IL-1β levels by immunoassays is vulnerable to analytical constraints (Cannon et al., 1988) which may have influenced these results. Likewise, IL-10 plasma levels were not altered in response to acute hypoglycemia in non-diabetic individuals (de Galan et al., 2003). A single study analyzed, amongst other pro-inflammatory cytokines, IL-8 plasma level response to hypoglycemia in non-diabetic individuals, showing a maximum response at 60 min after intravenous bolus administration of insulin, which is in line with the observed response for IL6 and TNF (Razavi Nematollahi et al., 2009).

3.8. Ex-vivo studies

Ex-vivo endotoxin-induced production of TNF, IL-1β, IL-6 and IL-10 by peripheral blood mononuclear cells (PBMCs) after acute hypoglycemia was assessed in T1DM patients and non-diabetic subjects (de Galan et al., 2003). Blood for cytokine measurements was drawn at baseline and at timely intervals during a hyperinsulinemic stepped hypoglycemic clamp. Whole blood was stimulated with lipopolysaccharide (LPS) and incubated for 24 h at 37 °C. Net LPS-stimulated cytokine production by PBMCs was calculated as the difference between the levels in the stimulated and the non-stimulated control tubes. Net TNF production was suppressed in T1DM patients at normoglycemia as compared to non-diabetic subjects, and down-regulated in response to hypoglycemia in both T1DM patients and in non-diabetic subjects. In contrast, IL-1β, IL-6, and IL-10 net production in response to LPS was not altered in any of the groups (de Galan et al., 2003).

A recent study showed that hypoglycemia enhanced pro-inflammatory cytokines (IL-6, TNF, IL-1β) production by LPS-stimulated PBMCs in non-diabetic subjects and T1DM patients with NAH (Ratter et al., 2017). Hypoglycemia had no effect on pro-inflammatory cytokine production in T1DM patients with IAH or on the production of the anti-inflammatory cytokine IL-10 in any of the groups. Moreover, hypoglycemia stimulates the production of pro-inflammatory cytokines, mainly TNF, by CD14 + cells and up-regulated PBMCs expression of integrin subunit alpha L (CD11a) and CX3C chemokine receptor 1 (CX3CR1) genes. This effect was associated with increased leukocyte demargination, as well as the expression of CD8 and CD16 genes, markers of cytotoxic effector potential (Ratter et al., 2017).

4. Discussion

From this review it is apparent that acute hypoglycemia increases total leukocyte number and biomarkers of inflammation (mainly IL-6) in both diabetic and non-diabetic subjects. Meanwhile, the apparent absence of a significant IL-10 change after hypoglycemic stimulus (de Galan et al., 2003; Ratter et al., 2017) indicates that an anti-inflammatory response is not elicited by acute hypoglycemia, at least soon after the hypoglycemia provoking stimulus.

Only one study did not show any stimulatory effect of acute hypoglycemia on inflammation (de Galan et al., 2003). In this study, plasma glucose concentration was gradually lowered, at hourly intervals, to 50.4 mg/dl (2.5 mmol/L) and blood sampling for the study of the inflammatory markers was performed as soon as the glucose nadir was achieved, as opposed to other studies that maintained the hypoglycemic clamp for at least one hour before sample collection (Ceriello et al., 2013a,b; Dotson et al., 2008; Gogitidze Joy et al., 2010; Joy et al., 2016; Ratter et al., 2017; Wright et al., 2010). Accordingly, this negative result could be due to the slow onset and the shorter duration of the hypoglycemic stimulus as inflammatory markers reach their maximum concentration 120 min after the hypoglycemic nadir (Ceriello et al., 2013a,b; Dotson et al., 2008; Galloway et al., 2000; Gogitidze Joy et al., 2010; Joy et al., 2016; Ratter et al., 2017; Razavi Nematollahi et al., 2009; Wright et al., 2010).

All current studies evaluating the effects of acute hypoglycemia on inflammatory markers in patients with diabetes were conducted in T1DM rather than T2DM patients (Ceriello et al., 2013a,b; de Galan et al., 2003; Galloway et al., 2000; Gogitidze Joy et al., 2010; Ratter et al., 2017; Wright et al., 2010). Possibly the main reason for this may be related to the significant evidence supporting the association between T2DM or insulin resistance and chronic low-grade inflammation. In fact, insulin resistance may be preceded by the activation of the

Table 3
Studies evaluating pro-inflammatory markers including T1DM patients and non-diabetic subjects.

Reference (Country)	Type of Study	Study Population	Study characteristics					Main outcomes
			Hypoglycemia induction protocol	Depth and duration of hypoglycemia	Measured inflammatory biomarkers	Laboratory methodology	Timed Blood collections	
Ratter et al., 2017 (The Nederlands)	Case-control	11 non-diabetic subjects, 10 T1DM patients with NAH, 10 T1DM patients with IAH (15 M/16F, 24.5 ± 5,3y) Mean A1c: not reported Hypoglycemia was avoided 24 h prior to the study	Hyperinsulinemic normoglycemic-hypoglycemic clamp	Mean glucose nadir of 2.6 mmol/L and maintained for 60 min	WBC count Cytokines production by PBMCs and CD14 + monocytes (IL1β, TNF, IL10, IL6, MCP-1) CD4, CD8, CD 14, CD 16, CD 56 CD11a , CX3CR1 expression in PBMCs	Flow citometry (WBC) ELISA (IL1β, TNF, IL10, IL6, MCP-1 production) qRT-PCRs (mRNA expression analysis)	At normoglycemia and after 1 h of hypoglycemia	Hypoglycemia increased leukocyte numbers in healthy controls and T1DM patients with NAH, but not in those with IAH ($p < 0.001$) Hypoglycemia enhanced PBMCs LPS stimulated production of IL-6, TNF, and IL1-β from non-diabetic and T1DM patients with NAH compared with normoglycemia ($p < 0.05$) Leukocytosis strongly correlated with adrenaline response ($p < 0.001$)
Ceriello et al., 2013a,b (Spain/Italy)	Case-control	15 T1DM patients (8 M, 23.2 ± 3.1y) Diabetic population presented normal autonomic tests, no hx of hypoglycemia unawareness and no major macro or microvascular complications Mean A1c: 8.1 ± 0.5% Hypoglycemia was avoided 5 days prior to the study Blood glucose was maintained between 4.4 and 7.2 mmol/L the night preceding the study by low-dose insulin infusion	Hyperinsulinemic hypoglycemia followed by normolycemia X Hyperinsulinemic hypoglycemia followed by hyperglycemia X Hyperinsulinemic hypoglycemia followed by hyperglycemia + V-itamin C X Hyperinsulinemic hypoglycemia followed by hyperglycemia + G-LP1 X Hyperinsulinemic hypoglycemia followed by hyperglycemia + V-itamin C + GLP1	Mean glucose nadir: 2.9 mmol/L -achieved after approximately 60 min and maintained for at least 60 min; rate of decline: 0.08 mmol/min	Plasma IL6	ELISA	Basal, +60, +120 min	Hypoglycemia significantly increased IL6 plasma levels ($p < 0.01$). The simultaneous infusion GLP-1 and vitamin C significantly attenuated this response ($p < 0.05$)
Ceriello et al., 2013a,b (Spain/Italy)	Case-control	30 type 1 diabetic subjects (15 M, 24.2 ± 2.1y) Diabetic population presented normal autonomic tests, no hx of hypoglycemia unawareness, no major macro or microvascular complications Mean A1c: 8.0 ± 0.4% Hypoglycemia was avoided 5 days prior to the study Blood glucose was maintained between 4.4 and 7.2 mmol/L the night preceding the study by low-dose insulin infusion	Hyperinsulinemic hypoglycemia X Hyperinsulinemic hypoglycemia + GL-P1 X Hyperglycemic clamp X Hyperglycemic clamp + GLP1	Mean glucose nadir: 2.9 mmol/L -achieved after approximately 60 min and maintained for at least 60 min; rate of decline: 0.08 mmol/min	Plasma IL6	ELISA	Basal, +60, +120 min	Hypoglycemia significantly increased IL6 plasma levels ($p < 0.01$). GLP1 significantly counterbalanced this effect ($p < 0.01$)

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Table 3 (*continued*)

Reference (Country)	Type of Study	Study Population	Study characteristics					Main outcomes
			Hypoglycemia induction protocol	Depth and duration of hypoglycemia	Measured inflammatory biomarkers	Laboratory methodology	Timed Blood collections	
Wright et al., 2010 (UK)	Case-control	16 non-diabetic subjects (6M), 28 (26.7–35) y X 16 T1DM patients (7M), 28 (25–37.5) y Diabetic population presented no hx of hypertension, macro or microvascular disease and no hx of hypoglycemia unawareness Mean A1c: 7.91 ± 0.92% Hypoglycemia was avoided 48 h prior to the study	Hyperinsulinemic normoglycemia X Hyperinsulinemic hypoglycemia	Glucose nadir: 2.58 ± 0.2 mmol/L lowered from 4.5 mmol/L over 20 min and hypoglycemia maintained for 60 min	Plasma IL6 and sCD40L Serum CRP CD 40 expression	Flow cytometry (CD40 expression) ELISA (sCD40L, IL6, CRP)	Basal, +45 min (during the clamp), +105 min (recovery), + 6 h, + 24 h	In non-diabetic subjects, hypoglycemia increased CD40 expression (p:0.009) and sCD40L levels (p:0.03) In T1DM patients, CD40 expression was elevated at baseline, but increments were also demonstrated after hypoglycemia (p:0.006). In non-diabetic subjects, basal and stimulated CRP levels were increased during hypoglycemia as compared to normoglycemia (p:0.02) IL6 levels rose in all experiments, with no significant difference between groups
Gogitidze Joy et al., 2010 (USA)	Case-control	35 non-diabetic subjects (19 M), 32 ± 2 y X 24 T1DM patients (12 M), 33 ± 3 y Diabetic population presented normal autonomic tests, no hx of hypoglycemia unawareness and no major macro or microvascular complications Mean A1c: 7.7 ± 0.2% Hypoglycemia was avoided 5 days prior to the study Blood glucose was maintained between 4.4 and 7.2 mmol/L the night preceding the study by low-dose insulin infusion	Hyperinsulinemic normoglycemia X Hyperinsulinemic hypoglycemia	Glucose nadir: 2.9 ± 0.1 mmol/L -achieved after aprox.. 30 min and maintained for 90 min; rate of decline: 0.08 mmol/min	Plasma IL6, TNF	LINCO research kits	Basal, +,60, +120 min (during the clamp)	IL-6 and TNF plasma levels were increased during hypoglycemia as compared with normoglycemia in both non-diabetic subjects and T1DM patients ($p < 0.05$).
de Galan et al., 2003 (The Nederlands)	Case-control	10 non-diabetic subjects (6M), 26.2 ± 5.3y X 9 T1DM patients (4M), 34.5 ± 2.9y Diabetic population with hx of hypoglycemia at least once a week, hypoglycemia awareness was not recorded Mean A1c: 7.2 ± 0.1% Hypoglycemia was avoided 3 days prior to the study	Hyperinsulinemic normoglycemia X Hyperinsulinemic hypoglycemia	Plasma glucose concentration was sequentially clamped at 5.0, 3.5 and 2.5 mmol/L at hourly intervals.	Plasma TNF,IL-1β, IL-6, IL-10. LPS-induced production of TNFα,IL-1β, IL-6, IL-10	RIA (TNF, IL1β) ELISA (IL-6, IL-10)	Basal,+ 90, +150, +210 min after insulin infusion (during the clamp)	In non-diabetic subjects, hypoglycemia downregulated LPS-induced production of TNF, but did not affect IL1β, IL-6 or IL-10. TNF production in T1DM was already suppressed at baseline (p :0.02) and fell in response to the hypoglycemia (p:0.04). IL6 and IL 10 levels were not measured in T1DM patients.

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Table 3 (*continued*)

Reference (Country)	Type of Study	Study Population	Study characteristics		Measured inflammatory biomarkers	Laboratory methodology	Timed Blood collections	Main outcomes
			Hypoglycemia induction protocol	Depth and duration of hypoglycemia				
Galloway et al., 2000 (UK)	Case-control	6 non-diabetic subjects (6M), 31.5 (24–39) y X 6 T1DM patients (6M), 36.5 (28–38) y No hx of diabetes related complications Mean A1c: 7.0%. Hypoglycemia was avoided 6 days prior to the study	Intravenous bolus of regular insulin (0,15 IU/kg)	Mean glucose nadir of 1.4 ± 0.2 mmol/L	Serum CRP	ELISA	Basal, +4h, +24 h after hypoglycemic nadir	Insulin-induced hypoglycemia induced a rise in CRP in T1DM patients and non-diabetic subjects ($p < 0.04$). Peak levels were observed at + 24 h.

M: males; y: years-old; hx: history; T1DM: type-1 diabetes mellitus; A1c: glycohemoglobin, U: units; NAW: normal awareness of hypoglycemia; IAH: impaired awareness of hypoglycemia, IL1β: interleucin 1β, IL6: interleucin 6, IL8: interleucin 8; CRP: C-reactive protein; sCD40L: soluble CD 40 ligand; TNF: tumor necrosis factor, WBC: white blood cells, GLP-1: glucagon-like peptide, PBMCs: peripheral blood mononuclear cells, MCP-1: monocyte chemoattractant protein-1, CD: cluster of differentiation, CX3CR1: CX3C chemokine receptor; qRT-PCR: quantitative real time polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; RIA: radioimmunoassay; UK: United Kingdom; USA: United States of America.

inflammatory system as evidenced by studies showing that monocyte-derived macrophages in the adipose tissue can secrete cytokines, such as IL-6 and TNF, leading to a pro-inflammatory state which, in turn, contributes to the development of insulin resistance (Eckel et al., 2005; Liu et al., 2016). Interestingly, a prospective observational study showed that T2DM-related low-grade chronic inflammation was associated with endothelial dysfunction and increased urinary albumin excretion, and T2DM patients with higher plasma CRP levels had increased risk of death (Stehouwer et al., 2002). Moreover, T2DM patients generally present high rates of other inflammation-related comorbidities such as obesity and dyslipidemia (B. M. Cheung and Li, 2012; Nguyen et al., 2011), which can potentially interfere with the assessment of the inflammatory response to the hypoglycemic stimulus. Notwithstanding, the study of the effects of acute hypoglycemia on inflammation markers in this population should not have been neglected.

As regards to the response of pro-inflammatory markers to acute hypoglycemia in T1DM patients as compared to non-diabetic subjects, few studies have concomitantly studied both groups under the same protocol (de Galan et al., 2003; Galloway et al., 2000; Gogitidze Joy et al., 2010; Ratter et al., 2017; Wright et al., 2010). It is worth noticing that high levels of circulating inflammatory markers have been associated with T1DM (Gomes et al., 2003; Scholin et al., 2004; Wedrychowicz et al., 2004). For instance, even T1DM patients with good glycemic control display higher baseline IL-6 and fibrinogen levels than age-matched individuals without diabetes (Snell-Bergeon et al., 2010). T1DM patients included in the current reviewed studies were of both sexes, did not present diabetes related complications or other comorbidities, and mean hemoglobin A1c levels varied from 6.9 to 8.1%. Two of these studies (Gogitidze Joy et al., 2010; Wright et al., 2010) compared the inflammatory response to hypoglycemia with a control sample exposed to a normoglycemic clamp, but did not directly compare the inflammatory response in T1DM with non-diabetic subjects. Revisiting the original data presented by Wright et al. (2010), the increase in IL6 plasma levels in non-diabetics subjects after hypoglycemia was higher than in T1DM patients (3.65 ± 0.27 pg/ml vs. 1.89 ± 0.325 pg/ml; $p = 0.002$). Gogitdze Joy and collaborators also found significant elevation of IL6 and TNF in both T1DM with NAH and non-diabetic subjects (Gogitidze Joy et al., 2010), but the original data were not available for between-groups comparison.

Recently, one study directly compared the inflammatory effects of hypoglycemia in T1DM patients with non-diabetics subjects (Ratter et al., 2017). T1DM patients were further divided in two groups according to their hypoglycemia awareness, namely, NAH or IAH. T1DM patients with IAH showed reduced PBMCs response to hypoglycemia as compared to T1DM with NAH and non-diabetic subjects. In addition, the increase in the number of leukocytes correlated positively with the adrenaline response to hypoglycemia in T1DM patients with NAH and non-diabetic subjects but not in patients with IAH (Ratter et al., 2017). The different inflammatory response to hypoglycemia in T1DM patients with and without impaired awareness of hypoglycemia is worth noticing. Considering that patients with NAH (associated with an intact sympathetic response) had a response similar to control subjects, this result indicates a likely role of adrenaline in the inflammatory response. Another study showed that adrenaline rise in response to acute hypoglycemia correlated with the increase of IL-8 and TNF levels (Razavi Nematollahi et al., 2009). Altogether these findings underscore the role of adrenaline in immune cell activation following hypoglycemia.

As early as 1983, Frier and collaborators suggested that changes in peripheral leukocyte counts were mediated, at least in part, by adrenergic mechanisms. Acute hypoglycemia was able to induce immediate increase in the number of lymphocytes in non-diabetic subjects, however this change was prevented under non-selective beta-blockade and in sympathectomized subjects (Frier et al., 1983). Previous data in animals suggests that reduced adrenaline synthesis may decrease the response of pro-inflammatory markers to endotoxin stimulation

(Giovambattista et al., 2000). In rats, plasma TNF concentrations were increased several fold after LPS treatment in control animals but not in phenylethanolamine-N-methyltransferase (PMNT) pre-treated animals, despite the observation of associated hypoglycemia in both groups. In this same study, ex-vivo experiments revealed that PBMCs from PMNT pre-treated animals secreted lower levels of TNF than PBMCs from control animals, while the addition of adrenaline to the medium containing PMBCs from PMNT pre-treated rats restored their secretory capacity. Nevertheless, the up-to-date literature have not yet evaluated the effect of sympatholytic drugs on the response of cytokines to acute hypoglycemia in humans.

Other potential mechanism involved in the inflammatory response following insulin stimuli is the activation of the HPA axis. A single study showed that higher levels of cortisol correlated to increased total leukocyte number and elevated IL-1β plasma levels, suggesting that cortisol was associated with cytokine and leukocyte stimulation during acute insulin-induced hypoglycemia (Razavi Nematollahi et al., 2009). Nevertheless, the precise relationship between pro-inflammatory cytokines and the HPA axis during hypoglycemia remains to be better investigated. Several cytokines have the ability to stimulate the HPA axis (Bernardini et al., 1990; Besedovsky and del Rey, 1987; Blalock, 1988; Turnbull and Rivier, 1999), reinforcing the classical concept of bidirectional communication between the immune and endocrine systems. Amongst them, IL-6 is of particular interest since it stimulates the HPA axis at several levels (Dotson et al., 2008). IL-6 can activate the pituitary-adrenal axis during immunological challenge in the absence of any hypothalamic input from CRH (Bethin et al., 2000). IL-6 receptors are present on the pituitary corticotrophins and on adrenocortical cells, consistent with the ability of IL-6 to bypass CRH in augmentation of adrenal function (Bethin et al., 2000). IL-6 injection into humans stimulates several anterior pituitary hormones, including ACTH, GH and prolactin, as well as cortisol, and glucagon, resulting in increased blood glucose levels (Tsigos et al., 1997b,a). As shown here, this cytokine clearly responds to acute hypoglycemia in humans. The inflammatory response to hypoglycemia is not limited to PBMCs. As previously discussed, elevation in CRP levels induced by acute hypoglycemia indicates hepatic response to increased circulating levels of cytokines, mainly IL-6 (Pepys and Hirschfield, 2003). Alike the classical counter-regulatory reaction to hypoglycemia, the activation of the inflammatory response seems to be a systemic rather than a localized reaction.

Regarding potential direct effects of insulin in the inflammatory response to acute hypoglycemia, in-vitro studies performed using PBMCs from non-diabetic subjects showed no TNF release after 24-hour incubation with insulin, whereas concomitant incubation with LPS and insulin resulted in a non-significant increase in TNF production by PBMCs (de Galan et al., 2003). In fact, previous evidence from clinical studies suggested an anti-inflammatory role of insulin (Chaudhuri et al., 2004; Cheung et al., 2006). Also, two of the studies that included a control hyperinsulinemic normoglycemic arm confirmed anti-inflammatory effects of insulin infusion (administered in the same rate as in the hyperinsulinemic hypoglycemic protocol) (Gogitidze Joy et al., 2010; Wright et al., 2010). Joy and collaborators observed that plasma TNF and IL6 levels decreased significantly during hyperinsulinemic euglycemia while Wright and collaborators showed a significant reduction in plasma soluble CD40 ligand levels during the hyperinsulinemic normoglycemic clamp compared to baseline.

The studies reviewed here addressed the acute and presumably transient nature of pro-inflammatory effects elicited by acute hypoglycemia. The long-term consequences of repeated hypoglycemia, particularly in the context of a pro-inflammatory state, have not been clearly defined yet. In addition to an increased pro-inflammatory response, it has been shown that acute moderate hypoglycemia also impairs fibrinolytic balance and decreases nitric oxide (NO)-mediated endothelial function (Joy et al., 2016). Recurrent hypoglycemic episodes could potentially influence the adaptive response of various systems (immune, neuroendocrine, sympathetic, endothelial) to acute hypoglycemia (Joy et al., 2015), further increasing the risk of associated morbidities, such as cardiovascular disease and neurocognitive decline.

The lack of a *meta*-analysis exploring the effect of hypoglycemia on different pro-inflammatory markers can be seen as the main limitation of the present review. Heterogeneity of the studies, i.e. different methodologies for cytokine measurement, distinct study populations, various protocols for hypoglycemia induction and unavailability of raw data on cytokine levels pre and post-hypoglycemia from most of the retrieved studies precluded us from performing this *meta*-analysis.

Insulin-induced hypoglycemia is a tool that could be better explored in psychoneuroimmunology research. The concomitant activation of the HPA axis and the sympathetic nervous system during acute hypoglycemia represents an interesting stress model to investigate the cross-talk between these adaptive systems and the immune response. Furthermore, inflammation has been recognized as a contributing factor for neuropsychiatric disorders, while hypoglycemia has been shown to sensitize microglial release of inflammatory mediators (Churchward et al., 2018). Accordingly, a better understanding of effect of these inflammatory mediators upon the regulation of hypothalamic activity could potentially help the development of novel therapies for these conditions.

5. Conclusion

In conclusion, acute hypoglycemia induces a pro-inflammatory state either after insulin intravenous bolus administration or hyperinsulinemic clamp in both diabetic and non-diabetic subjects. Sympathetic mechanisms are likely to play a role in this inflammatory response. However further studies are needed to unveil the complex interactions among the pro-inflammatory response, the neuroendocrine and the sympathetic nervous systems in response to acute hypoglycemia.

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